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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:

Rea et al.

Serial No.: 10/666,778

Filed: September 18, 2003

For: THE USE OF GENES ENCODING MEMBRANE TRANSPORTER PUMPS TO STIMULATE THE PRODUCTION OF SECONDARY METABOLITES IN BIOLOGICAL CELLS

Confirmation No.: 8721

Examiner: R. Kallis, Ph.D.

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BRIEF ON APPEAL

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(1) REAL PARTY IN INTEREST

The real parties in interest in the present pending appeal are Vlaams Interuniversitair Instituut Voor Biotechnolgie VZW and Valtion Teknillinen Tukimuskeskus, assignees of the pending application as recorded with the United States Patent and Trademark Examiner on May, 2005, at Reel 016715, Frame 0520.

(2) RELATED APPEALS AND INTERFERENCES

Neither the Appellants, the Appellants' representative, nor the Assignees are aware of any pending appeal or interference which would directly affect, be directly affected by, or have any bearing on the Board's decision in the present pending appeal.

(3) STATUS OF THE CLAIMS

Claim 14 was cancelled without prejudice or disclaimer.

Claims 1-13, 16-19, and 22-24 stand rejected.

Claims 15, 20, and 21 are allowed.

The rejections of claims 1-13, 16-19, and 22-24 are being appealed.

(4) STATUS OF AMENDMENTS

The appellants' amendments filed November 21, 2008 have been entered.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 recites "a method of inducing or enhancing production of at least one secondary metabolite by plant cells, said method comprising: transforming plant cells with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter; wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells; selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cells." Support for "a method of inducing or enhancing production of at least one secondary metabolite by plant cells" can be found at, for example, page 5, lines 16-22. Support for "transforming plant cells with an expression vector comprising an expression

cassette comprising a gene encoding an ABC-transporter" can be found at, for example, page 10, lines 7-12 of the Specification. Support for "wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold," can be found at, for example, page 10, lines 18-26 of the Specification. Support for "wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 5, lines 22-6, and page 10, lines 7-12 of the Specification. And support for "selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cells" can be found at, for example, page 14, lines 2-4. Support for these elements combined in the manner recited in claim 1 can be found at, for example, page 13, line 28 through page 14, line 4 of the Specification.

Independent claim 7 recites, "A method of stimulating the production of secondary metabolites by plants, the method comprising: transforming said plants with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter; wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells; selecting transformed plants based upon enhanced production of secondary metabolites; and propagating such selected transformed plants." Support for "a method of stimulating the production of at least one secondary metabolite by plant cells" can be found at, for example, page 5, lines 16-22. Support for "transforming said plants with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter" can be found at, for example, page 10, lines 7-12 of the Specification. Support for "wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold," can be found at, for example, page 10, lines 18-26 of the Specification. Support for "wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 5, lines 22-6, and page 10, lines 7-12 of the Specification. And support for "selecting transformed plants based upon enhanced production of secondary metabolites; and propagating such selected transformed plant cells" can be found at, for example, page 14, lines 2-4. Support for these elements combined in the manner recited in claim 1 can be found at, for example, page 13, line 28 through page 14, line 4 of the Specification.

Independent claim 10 recites, "A transgenic plant cell culture displaying an enhanced production of at least one secondary metabolite, wherein said transgenic plant cell is transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter; wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells."

Support for "A transgenic plant cell culture displaying an enhanced production of at least one secondary metabolite" can be found at, for example, page 4, lines 10-12, and page 12, lines 11-13 of the Specification. Support for "wherein said transgenic plant cell is transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter" can be found at, for example, page 10, lines 7-12 of the Specification. Support for "wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold," can be found at, for example, page 10, lines 18-26 of the Specification. Support for "wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 5, lines 22-6, and page 10, lines 7-12 of the Specification. Support for these elements combined in the manner recited in claim 10 can be found at, for example, page 10, lines 3-6 of the Specification.

Independent claim 12 recites, "A transgenic plant material selected from the group consisting of a plant, plant cells, plant seeds and plant progeny, said transgenic plant material capable of an enhanced production of at least one secondary metabolite, said transgenic plant material transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter; wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells." Support for "A transgenic plant material selected from the group consisting of a plant, plant cells, plant seeds and plant progeny" can be found at, for example, page 12, line 37 through page 14, line 4 and page 19, lines 20-31 of the Specification. Support for "said transgenic plant material capable of an enhanced production of at least one secondary metabolite" can be found at, for example, page 19, lines 20-23, page 4, lines 10-12, and page 12, lines 11-13 of the Specification. Support for "said transgenic plant

material transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter" can be found at, for example, page 10, lines 7-12 of the Specification. Support for "wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold," can be found at, for example, page 10, lines 18-26 of the Specification. Support for "wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 5, lines 22-6, and page 10, lines 7-12 of the Specification. Support for these elements combined in the manner recited in claim 12 can be found at, for example, page 10, lines 3-6, page 12, line 37 through page 14, line 4, and page 19, lines 20-31 of the Specification.

Independent claim 16 recites, "A process for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite, said process comprising: transforming a plant cell with an expression cassette comprising a gene encoding an ABC-transporter; wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells; and selecting transformed plant cells exhibiting enhanced transport of said at least one secondary metabolite into a vacuole." Support for "A process for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite" can be found at, for example, page 4, lines 10-12, page 12, lines 11-13, page 12, line 37 through page 14, line 4 and page 19, lines 20-31 of the Specification. Support for "said process comprising: transforming a plant cell with an expression cassette comprising a gene encoding an ABC-transporter" can be found at, for example, page 10, lines 7-12 of the Specification. Support for "wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold," can be found at, for example, page 10, lines 18-26 of the Specification. Support for "wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 5, lines 22-6, and page 10, lines 7-12 of the Specification. Support for "selecting transformed plant cells exhibiting enhanced transport of said at least one secondary metabolite into a vacuole" can be found at, for example, page 16, line 20 through page 17, line 22 of the Specification. Support for these elements combined in the manner recited in claim 16 can be found at, for example, page 10, lines 3-6, page 12, line 37 through page 14, line 4, and

page 19, lines 20-31 of the Specification.

Independent claim 19 recites "An isolated polynucleotide useful for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite, said isolated polynucleotide comprising: a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells, and a second sequence of nucleotides bases, operatively positioned with respect to said first sequence, constituting a means for promoting expression of said first sequence. Support for "An isolated polynucleotide useful for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite, can be found at, for example, page 20, lines 12-28 of the Specification. Support for "said isolated polynucleotide comprising: a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells" can be found at, for example, page 10, lines 7 through page 12, line 6 and page 20, lines 12-28 of the Specification. Support for "and a second sequence of nucleotides bases, operatively positioned with respect to said first sequence, constituting a means for promoting expression of said first sequence," can be found at, for example, page 5, line 27 through page 7, line 15 of the Specification. Support for these elements combined in the manner recited in claim 16 can be found at, for example, page 10, lines 3-6, page 12, line 37 through page 14, line 4, and page 19, lines 20-31 of the Specification.

Independent claim 22 recites "A method of inducing or enhancing production or cellular secretion of at least one endogenous secondary metabolite by a plant cell, the method comprising: transforming the plant cell with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter, wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold, and functions to transport at least one secondary metabolite in plant cells; wherein the secondary metabolite is an endogenous metabolic product of the plant cell, and is transported from the cell to the extracellular space; wherein the amount of secondary metabolite recoverable from the cell is increased; selecting a transformed plant cell having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cell. Support for "A method of inducing or enhancing production or cellular secretion of at least one endogenous secondary

metabolite by a plant cell, page 5, lines 16-26 of the Specification. Support for "transforming the plant cell with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter, wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold, and functions to transport at least one secondary metabolite in plant cells" can be found at, for example, page 10, lines 7-12, page 10, lines 18-26, and page 5, lines 22-26 of the Specification. Support for "wherein the secondary metabolite is an endogenous metabolic product of the plant cell, and is transported from the cell to the extracellular space," can be found at, for example, page 9, lines 1-22, and page 13, line 25 through page 14, line 12 of the Specification. Support for these elements combined in the manner recited in claim 16 can be found at, for example, page 10, lines 3-6, page 12, line 37 through page 14, line 4, and page 19, lines 20-31 of the Specification.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1-13, 16-19, and 22-24 are unpatentable under 35 U.S.C. § 103(a) over Rea *et al.* (WO 98/21938) (hereinafter "Rea")?

B. Whether claims 1-13, 16-19, and 22-24 are unpatentable under 35 U.S.C. § 103(a) over Theodoulou (Biochmica et Biophysica Acta; 2000, 1465, pp. 79-103) (hereinafter "Theodoulou") in view of Dudler *et al.* (Journal of Biological Chemistry; 25 March 1992, Vol. 267, No. 9, pp. 5582-5588) (hereinafter "Dudler") and Sidler *et al.* (The Plant Cell, 10:1623-1636) (hereinafter "Sidler")?

(7) ARGUMENT

A. Claims 1-13 and 16-19 and 22-24 are not obvious under 35 U.S.C. § 103(a) in view of Rea.

Alleged basis of rejection

Claims 1-13, 16-19, and 22-24 stand rejected under 35 U.S.C. § 103(a) as assertedly being unpatentable over Rea. Rea describes the transport of secondary metabolites by an ABC transporter into the vacuole. The Examiner Action of February 26, 2008, states that "[o]ne of ordinary skill would have been motivated by the teachings of Rea that both alkaloid and a pigment could be targeted to a plant vacuole for increased transport using a plant GS-X ABC transporter, [and] would have a reasonable expectation of success in screening for increases in either secretion or production into the vacuole of either anthocyanin or medicarpin...".

The Final Examiner Action of September 22, 2008, asserts, at page 4, that "[w]hen a metabolite is sequestered away from the cytosol it is protected from oxidation and thus the secretion to the vacuole will result in enhanced production as recited in the claim." The Examiner further cites ¶ [0043] of the appellants' Specification, which teaches that "[a]n enhanced production of a secondary metabolite can result in a higher level of secondary metabolites in the plant, for example in the vacuole."

In the Advisory Actions of January 2, 2009 and February 4, 2009, the Examiner further argues that this section of ¶ [0043] "defines production as an increased level of detection in the vacuole."

Rea does not teach selecting transformed plant cells having an induced or enhanced production of a secondary metabolite.

Claims 1, 7, and 22 each require a selection step where a cell having increased production of a secondary metabolite is selected and then propagated. Specifically, claim 1 recites "selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cells;" claim 7 recites "selecting transformed plants based upon enhanced production of secondary metabolites; and propagating

such selected transformed plants;" and claim 22 recites "selecting a transformed plant cell having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cell."

Rea does not teach the selecting cells on the basis of increased production or propagating such cells once they have been selected. Consequently, claims 1, 7, 22, and the claims depended therefrom cannot be considered obvious over Rea as neither Rea, nor the inferences and creative steps a person of ordinary skill in the art would have employed at the time of the invention, teach or suggest selecting cells on the basis of increased production or propagating such cells once they have been selected.

Rea does not teach enhanced production of a secondary metabolite by the plant

To establish a *prima facie* case of obviousness, the prior art itself or "the inferences and creative steps that a person of ordinary skill in the art would [have] employ[ed]" at the time of the invention are to have taught or suggested the claim elements. Additionally, the Examiner must determine whether there is "an apparent reason to combine the known elements in the fashion claimed by the patent at issue." *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1740-1741, 167 L.Ed.2d 705, 75 USLW 4289, 82 U.S.P.Q.2d 1385 (2007). Further, rejections on obviousness grounds "cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *Id* at 1741, quoting *In re Kahn*, 441, F.3d 977, 988 (Fed. Cir. 2006). "Often, it will be necessary for a [fact finder] to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed . . . . To facilitate review, this analysis should be made explicit." *Id.* Furthermore, to establish a *prima facie* case of obviousness there must have been a reasonable expectation of success. M.P.E.P. § 2143.02. Underlying the obvious determination is the fact that statutorily prohibited hindsight cannot be used. *KSR*, 127 S.Ct. at 1742.

Appellants respectfully submit that Rea does not teach enhanced production of a

secondary metabolite by a plant resulting from expression of an ABC transporter comprising a Walker A box, a Walker B box, and a Nucleotide Binding Fold as required in independent claims 1, 7, 10, 12, 16, and 22. Applicants do not understand what was meant by the Examiner's use of "production into the vacuole."

"Production" is defined in paragraph [0043] of the as filed Specification, to wit: "to enhance the production" means that the level of a given metabolite in a transformed plant is enhanced by at least 20% relative to the level of the secondary metabolite in the untransformed plant. This definition is consistent with the common usage of the term "production" in the art. Further in paragraph [0043], it is recited that "[a]n enhanced production of a secondary metabolite can result in a detection of a higher level of secondary metabolites in the plant, for example in the vacuole." This statement however does not give "production" a contrary meaning to its common usage or its definition in the Specification. For example, a plant exhibiting enhanced production of a secondary metabolite may also exhibit a higher level of that metabolite in the vacuole. It does not necessarily follow, however, that simply because a transformed plant exhibited a higher level of a secondary metabolite in a vacuole, that the plant exhibits enhanced production of that metabolite. Thus, contrary to the conclusion reached by the Examiner, increased transport into the vacuole is not necessarily indicative of increased production as defined by appellants' Specification.

Original claim 4 further discredits the Examiner's view regarding increased production as defined by the Specification. Original claim 4 recites "[t]he method according to claim 1 wherein the induction or enhancement of the production of at least one secondary metabolite by plant cells results from enhancing the transport of said secondary metabolite into a vacuole." Claim 4 thus indicates that increased production can result from transport into the vacuole. This is contrary to the Examiner's interpretation that increased transport into the vacuole <u>is</u> increased production. If, as asserted by the Examiner, increased transport into the vacuole <u>is</u> increased "production," claim 4 would have no meaning. Increased levels of the secondary metabolite in the vacuole would then mean that increased production had already been accomplished and therefore increased production could not then <u>result from</u> the increased sequestration in the vacuole as required by claim 4.

Claim 4 is meant to describe the situation where there is a feedback inhibition loop in the cytosol wherein levels of the secondary metabolite in the cytosol prevent any further production of the secondary metabolite. If levels of the secondary metabolite in the cytosol are lowered by transport into the vacuole, the feedback inhibition would be ameliorated and production of the secondary metabolite could again proceed thus increasing the overall production of the secondary metabolite.

Rea does not describe the resulting increased production of the secondary metabolite in the cell. Rea only describes a situation where an increased level of a vacuolar transporter leads to increased transport of a secondary metabolite into the vacuole. Rea does not indicate anywhere that intracellular levels of the secondary metabolite are thus increased, only that they are more efficiently moved from one location within the cell to another.

In summary, increased levels of a secondary metabolite in a vacuole are not equivalent to increased production of the secondary metabolite as borne out by appellants' Specification and original claim 4. Further, neither Rea, nor the inferences and creative steps a person of ordinary skill in the art would have employed at the time of the invention, teach or suggest that increased levels of a ABC transporter would lead to increased production of a secondary metabolite as claimed.

Rea does not teach a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells

Independent claim 19 requires "a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells." Rea does not describe such a sequence of nucleotide bases. As described *supra*, Rea teaches nucleotides sequences encoding proteins that have the ability to move a substrate from the cytosol to the vacuole. However, Rea does not describe that, as a consequence of that particular movement of the substrate, increased levels of a secondary metabolite are produced. Thus Rea cannot be said to teach such a sequence. Consequently, claim 19 cannot be considered obvious over Rea as neither Rea, nor the inferences and creative steps a person of ordinary skill in the art would have employed at the time of the invention, teach or suggest a sequence of

nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells.

For at least the foregoing reasons, appellants respectfully request the withdrawal of the rejections of 1, 7, 10, 12, 16, 19, and 22, and the claims dependent therefrom (claims 2-6, 8, 9, 11, 13, 17, 18, 23, and 24) under 35 U.S.C. § 103(a) in view of Rea.

B. Claims 1-13 and 16-19 and 22-24 are not obvious under 35 U.S.C. § 103(a) over Theodoulou in view of Dudler and Sidler.

Alleged basis of rejection

Claims 1-13, 16-19, and 22-24 stand rejected under 35 U.S.C. § 103(a) as assertedly being unpatentable over Theodoulou in view of Dudler and Sidler. Theodoulou assertedly teaches that ABC transporter genes from plants have a strong similarity to MDR proteins from other species and suggests a role of the plant homologs in secretion or sequestering of *vinca* alkaloid and the alkaloid taxol and suggests a strategy for screening transformed plants and plant cells for determining the specific transport function. Examiner Action of February 14, 2007, at page 4.

The Examiner assets that Dudler teaches an MDR like gene (*At*PGP1) from *Arabidopsis*, having characteristics of ABC transporters and suggests testing to identify substrates. Examiner Action of July 31, 2007, at page 5. The Examiner further asserts that Sidler teaches transformation of *Arabidopsis* with the *At*PGP1 gene of Dudler where overexpression (*i.e.* secretion to the plasmalemma) resulted in an increase in hypocotyl length and strongly suggests a transport process. *Id.* The Examiner further asserts that it is well known in the art that hypocotyl elongation is a response to auxin, which is a secondary metabolite. Examiner Action of February 26, 2008, at pages 7 and 8.

In the Examiner Action of February 26, 2008, the Examiner asserts, on pages 6 and 7, that "[i]t would have been obvious at the time of Applicant's filing to take any ABC transporter gene that encoded a protein that had similar structural motifs to the human MDR ABC transporter and

test for induced or enhanced production or secretion of any secondary metabolite such as *vinca* alkaloid or taxol to determine the function of the plant MDR homologue."

The Examiner further asserts that the state of the art suggests and was actively involved in isolating, characterizing, *i.e.* selecting for expression above wildtype including vacuolar accumulation, and transforming said ABC transporter genes into plants. *Id.* at pages 7 and 8. The Examiner goes on to assert that "increased expression of AtPGP1 and the increase in hypocotyls elongation observed in Sidler, combined with the knowledge of the structure of AtPGP1 as an ABC transporter, it would be well understood by one of ordinary skill that increased expression of AtPGP1 would result in the increased transport of a secondary metabolite.

Theodoulou in view of Dudler and Sidler does not teach selecting transformed plant cells having an induced or enhanced production of a secondary metabolite.

Claims 1, 7, and 22 each require a selection step where a cell having increased production of a secondary metabolite is selected and then propagated. Specifically, claim 1 recites "selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cells;" claim 7 recites "selecting transformed plants based upon enhanced production of secondary metabolites; and propagating such selected transformed plants;" and claim 22 recites "selecting a transformed plant cell having an induced or enhanced production of at least one secondary metabolite; and propagating such selected transformed plant cell."

The references do not teach the selecting cells on the basis of increased production or propagating such cells once they have been selected. Consequently, claims 1, 7, 22, and the claims depended therefrom cannot be considered obvious over the references as neither the references, nor the inferences and creative steps a person of ordinary skill in the art would have employed at the time of the invention, teach or suggest selecting cells on the basis of increased production or propagating such cells once they have been selected.

For at least the foregoing reasons, appellants respectfully request the withdrawal of the rejections of 1, 7, 10, 12, 16, 19, and 22, and the claims dependent therefrom (claims 2-6, 8, 9, 11, 13, 17, 18, 23, and 24) under 35 U.S.C. § 103(a) in view of the references.

Theodoulou in view of Dudler and Sidler does not teach enhanced production of a secondary metabolite

Applicants respectfully submit that the claims cannot be obvious over Theodoulou in view of Dudler and Sidler (hereinafter "the references") as the references do not teach or suggest each and every claim element. Specifically, the independent claims are directed to "inducing or enhancing production of at least one secondary metabolite" (claim 1); "stimulating the production of secondary metabolites" (claim 7); "a transgenic plant cell culture displaying an enhanced production of at least one secondary metabolite" (claim 10); "transgenic plant material capable of an enhanced production of at least one secondary metabolite" (claim 12); "a plant cell exhibiting an enhanced production of at least one secondary metabolite" (claim 16); and "a transformed plant cell having an induced or enhanced production of at least one secondary metabolite." Thus, all of the rejected independent claims recite, in one form or another, that there is induced or enhanced production of a secondary metabolite. Further, each of these claims ties that induced or enhanced production to the presence of an exogenously provided ABC transporter. Applicants assert that the references do not teach induced or enhanced production of secondary metabolite tied to the expression of an exogenously provided ABC transporter.

At most, the references relate to the concept that ABC transporters are capable of transporting secondary metabolites across membranes. Theodoulou suggests screening MDR like genes for determining the specific transport function. Dudler suggests that AtPGP1 is a transporter and further suggests testing for determining what the substrates of AtPGP1 are. Sidler, according to the Examiner, strongly suggests a transport function for a hypocotyl length-increasing compound. Furthermore, Sidler specifically refers separately to the transport (*i.e.* secretion) of the unknown hypocotyl length-increasing compound and the production of that compound. As Sidler states in the portion of the discussion entitled, "How May AtPGP1 Affect Hypocotyl Length?":

> An attractive hypothesis is that the AtPGP1 protein is involved in the transport of such a signal. A conceivable function of AtPGP1 would therefore be the export of a hormone-like compound from the shoot apical region that would regulate hypocotyl cell length. The production of this compound would be under the control of the light fluence rate perceived by the cotyledons and possibly other, yet unknown factors, whereas its export would be mediated by the constitutive AtPGP1 exporter. Sidler *et al.*, at 1631.

This passage unarguably shows that Sidler does not suggest the ABC transporter described by their work is involved in the production of a secondary metabolite. In fact, Sidler specifically teaches away from a role for AtPGP1 in the production of the secondary metabolite and ascribes that function to other factors while reserving export for AtPGP1.

Dudler provides further direction to one of skill in the art that plant ABC transporters are involved in transport, as opposed to production. With respect to members of the ABC transporter family, in general, Dudler hypothesizes, "it seems likely that they are all involved in some transport process." Dudler, at 5888. With regard to mammalian P-glycoproteins, they state, "[t]he normal function of these proteins is not known, but it has been hypothesized that it may be the elimination of toxic metabolic or xenobiotic substances." *Id.*, at 5888. Finally, Dudler *et al.* can only conclude from their research that plant ABC transporters are likely to also be involved in transport processes. "[T]he fact that the *Arabidopsis* protein exhibits the highest sequence similarity to the mammalian P-glycoproteins may suggest that functional aspects are also conserved. Thus, the *atpgp1* gene product may be involved in the extrusion of toxic metabolic and xenobiotic compounds from cells." *Id.*, at 5888.

Further, while the references may suggest a role in transport or secretion for ABC transporters, none of these teachings indicates that an ABC transporter may induce or enhance the actual production of a secondary metabolite. As delineated by appellants' specification, production and secretion are separate processes. "Enhanced production" is defined at paragraph [0043] of the Specification as published; to wit:

> By the term "to enhance the production" it is meant that the level of one or more metabolites may be enhanced by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or at least 100% relative to the untransformed plant which was used to

> transform with an expression vector comprising an expression cassette further comprising a gene coding for a transporter or an ABC-transporter.

"Enhanced secretion" is defined at paragraph [0032] of the Specification as published; to wit:

> An "enhanced secretion of at least one secondary metabolite" means that there exists already a detectable secretion of the secondary metabolite(s) in the extracellular medium of the plant cell culture and that an increase of the secondary metabolite(s) can be measured by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more than 90% compared to basal secretion by the untransformed plant cell culture. An "enhanced secretion" does not necessarily mean that there is a higher production, it can also mean that there exists the same level of production but that the secretion is enhanced.

Thus, the Specification clearly delineates production and secretion as separate processes. Applicants respectfully submit that the sections of paragraph [0043] cited by the Examiner indicate that increased production may lead to increased secretion, but also indicate that increased secretion can happen in the absence of increased production.

Applicants further note that claim 1 recites "selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite;" claim 7 recites "selecting transformed plants based upon enhanced production of secondary metabolites;" and claim 22 recites "selecting a transformed plant cell having an induced or enhanced production of at least one secondary metabolite." Applicants respectfully submit that the references do not teach selecting the transformed cells by any of the criteria provided for in these claims, to wit: induced or enhanced production of a secondary metabolite. Theodolou is a review article and provides no teaching of how to select transformed cells based on production. Sidler teaches the selection of transgenic plants by kanamycin resistance (page 1634, column 1, 1st pargraph). Dudler teaches the isolation of the AtPGP gene, but never produces cells expressing the gene and therefore engages in no selection of transformed cells. As such, applicants respectfully submit that the references do not teach selecting transformed cells based on production.

Applicants respectfully submit there is not a single demonstration or a suggestion in the cited art that teaches a skilled person that, in order to obtain increased production of a secondary metabolite in a plant, it suffices to transform the plant with an ABC transporter. Restated, there

is no teaching or suggestion that transporting secondary metabolites will obviously result in their increased production. Applicants note that secondary metabolites are often toxic for plants. However, it has not been demonstrated or suggested that removing toxic compounds via ABC transporters will result in a higher production of such toxic compounds.

Theodoulou in view of Dudler and Sidler does not teach a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells

Independent claim 19 requires "a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells." The references do not describe such a sequence of nucleotide bases. As described *supra*, the references teach a nucleotides sequences that leads to hypocotyl elongation. However, the references do not describe that, as a consequence of transfection of such nucleotide sequences, increased levels of a secondary metabolite are produced. Thus the references cannot be said to teach such a sequence. Consequently, claim 19 cannot be considered obvious over the references as neither the references, nor the inferences and creative steps a person of ordinary skill in the art would have employed at the time of the invention, teach or suggest a sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells.

Declaration of Alain Gossens

Applicants note that the PTO is subject to the Administrative Procedures Act and thus their conclusions must be supported by substantial evidence. *See, e.g.*, Dickinson v. Zurko 527 U.S. 150 (1999); and In re Sang-Su Lee, 277 F.3d 1338 (C.A.Fed.,2002).

> As applied to the determination of patentability *vel non* when the issue is obviousness, "it is fundamental that rejections under 35 U.S.C. § 103 must be based on evidence comprehended by the language of that section." In re Grasselli, 713 F.2d 731, 739, 218 USPQ 769, 775 (Fed.Cir.1983). The essential factual evidence on the issue of obviousness is set forth in Graham v. John Deere Co., 383 U.S. 1, 17-18, 86 S.Ct. 684, 15 L.Ed.2d 545, 148 USPQ 459, 467 (1966) and extensive ensuing precedent. In re Sang Su Lee at 1342 -1343.

Applicants submit that determinations of a reasonable expectation of success and unexpected results are part of the factual inquiries underlying a legal determination of obviousness and "must be based on objective evidence of record." *Id.* at 1343; *see also*, In re Kotzab (217 F.3d 1365, 1369 (Fed, Cir. 2000)) and In re Merck & Co., Inc. (800 F.2d 1091, 1097 (Fed. Cir. 1986)). Applicants note that the evidence of record in the present application consists of the materials supplied in applicants IDS, the Declaration of Dr. Alain Goossens, as well as the references which are cited by the Examiner as basis for the obviousness rejection of claims.

Applicants submit that the references, in and of themselves, make no determination as to whether the claims of the present application embody unexpected results. Any interpretation, argument, or finding by the Examiner as to what those references may or may not teach in relation to the claims at issue is based on the subjective belief of the Examiner and thus cannot be considered evidence of record.

In contrast, the sworn Declaration of Dr. Alain Goossens, evidence of record which must be considered by the Patent and Trademark Examiner (In re Sullivan, 498 F.3d 1345, 1351 (Fed. Cir. 2007)) makes clear statements regarding the unexpected nature of the results embodied in the pending claims. Declaration of Dr. Alain Goossens at point 6. In addition, and as noted in the Declaration of Alain Goossens, these statements are based upon experimental data that is also, by virtue of being included in the Declaration, evidence of record. Applicants note that the Examiner has provided no evidence of record, other than the references cited above, to rebut the statement of unexpected results presented in the Declaration of Dr. Alain Goossens. As noted *supra*, the above cited references do not teach that increased production of a secondary metabolite would be an expected result of transforming a plant cell with an ABC transporter.

Thus, when viewed as a whole (as required under M.P.E.P. § 2141.02(VI)), the evidence of record, through clear and unambiguous statements therein, weighs for a finding that the pending claims embody unexpected results.

In view of the foregoing, applicants respectfully submit that there is a clear legal and factual deficiency in the rejection of the present claims as the evidence of record, which must form the basis of any agency decision, provides a conclusion of non-obviousness through a direct finding of unexpected results and no reasonable expectation of success. Furthermore, applicants

submit that there is no other evidence of record that makes direct statements contravening the explicit statements of the evidence regarding unexpected results.

Impropriety of the Examiner's Reliance on Auxin Being Known as the Factor in Hypocotyl Elongation

In the Examiner Action of February 26, 2008, the Examiner asserts that "the prior art (Sidler) suggests that AtPGP1 transports a regulator involved in light dependent hypocotyls elongation, and speculates that that it may be a peptide. However, one must also consider what is well known in the art, that hypocotyl elongation is a response to auxin, which is a secondary metabolite."

Applicants note that the Examiner has provided no evidence of its assertion that one of ordinary skill in the art would have concluded that the hypocotyl elongation described in Sidler is a response to auxin. Applicants note that the Examiner is subject to the Administrative Procedures Act and all conclusions must be based upon evidence of record. *See, e.g.*, Dickinson v. Zurko 527 U.S. 150 (1999); and In re Sang-Su Lee, 277 F.3d 1338 (C.A.Fed.,2002). Without evidence to support the assertion of the Examiner, such assertions should be ignored as merely Examiner argument. Further, the Sidler teaches that the substrate of the AtPGP1 transporter is likely a peptide. This is in direct contravention to the unsupported assertion of the Examiner that one of ordinary skill in the art would have considered auxin as the substrate.

Even if auxin were transported by AtPGP1 and was responsible for the hypocotyl elongation (which the applicants respectfully submit the Examiner has not shown), there is nothing in the art cited that would suggest that the increased hypocotyl elongation is due to increased production of auxin as opposed to increased secretion. Applicants respectfully submit that better secretion of auxin, absent increased production, might be responsible for the increased hypocotyl elongation described by Sidler. However, the prior art cited provides no indication, one way or another, in regards to increased production and any assertion that increased production has indeed occurred is no more than unsupported speculation on the part of the Examiner.

For at least the foregoing reasons, appellants respectfully request the withdrawal of the rejections of 1, 7, 10, 12, 16, 19, and 22, and the claims dependent therefrom (claims 2-6, 8, 9, 11, 13, 17, 18, 23, and 24) under 35 U.S.C. § 103(a) over Theodoulou in view of Dudler and Sidler.

CONCLUSION

For at least the foregoing reasons, appellants respectfully submit that all pending claims are in condition for immediate allowance. Consequently, appellants respectfully request that the rejections of claims 1-13, 16-19, and 22-24 under 35 U.S.C. 103(a) be withdrawn, and the rejected claims allowed.

(8) CLAIMS APPENDIX

1. A method of inducing or enhancing production of at least one secondary metabolite by plant cells, said method comprising:

transforming plant cells with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter;

wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold;

wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells;

selecting transformed plant cells having an induced or enhanced production of at least one secondary metabolite; and

propagating such selected transformed plant cells.

2. The method according to claim 1 wherein the secondary metabolites are alkaloids.

3. The method according to claim 1 wherein the ABC-transporters are of plant, fungal, or mammalian origin.

4. The method according to claim 1 wherein the induction or enhancement of the production of at least one secondary metabolite by plant cells results from enhancing the transport of said secondary metabolite into a vacuole.

5. The method according to claim 4 wherein the secondary metabolites are alkaloids.

6. The method according to claim 4 wherein the ABC-transporters are of plant, fungal, or mammalian origin.

7. A method of stimulating the production of secondary metabolites by plants, the method comprising:

transforming said plants with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter;

wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and

wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells;

selecting transformed plants based upon enhanced production of secondary metabolites; and

propagating such selected transformed plants.

8. The method according to claim 7 wherein the secondary metabolites are alkaloids.

9. The method according to claim 7 wherein the ABC-transporters are of plant, fungal, or mammalian origin.

10. A transgenic plant cell culture displaying an enhanced production of at least one secondary metabolite, wherein said transgenic plant cell is transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter;

wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and

wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells.

11. The transgenic plant cell culture of claim 10 further characterized in having
(1) an increased vacuolar localization of said at least one secondary metabolite, or
(2) a secretion or an increased secretion of said at least one secondary metabolite.

12. A transgenic plant material selected from the group consisting of a plant, plant cells, plant seeds and plant progeny, said transgenic plant material capable of an enhanced production of at least one secondary metabolite, said transgenic plant material transformed with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter;

wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold; and

wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells.

13. The transgenic plant material of claim 12 further characterized in having an increased vacuolar localization of said at least one secondary metabolite.

15. An isolated polynucleotide sequence comprising a sequence having at least 91% identity to the polynucleotide sequence of SEQ ID NO:1;

wherein the isolated polynucleotide sequence induces or enhances production of at least one secondary metabolite in plants.

16. A process for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite, said process comprising:

transforming a plant cell with an expression cassette comprising a gene encoding an ABC-transporter;

wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold;

wherein said ABC-transporter functions to transport at least one secondary metabolite in plant cells; and

selecting transformed plant cells exhibiting enhanced transport of said at least one

secondary metabolite into a vacuole.

17. A plant cell produced by the process of claim 16.

18. A transgenic plant including the plant cell of claim 17.

19. An isolated polynucleotide useful for producing a plant cell exhibiting an enhanced production of at least one secondary metabolite, said isolated polynucleotide comprising:

a first sequence of nucleotide bases constituting a means for inducing or enhancing production of at least one secondary metabolite in plants or plant cells, and

a second sequence of nucleotides bases, operatively positioned with respect to said first sequence, constituting a means for promoting expression of said first sequence.

20. The isolated polynucleotide sequence of claim 15, wherein the isolated polynucleotide sequence comprises the polynucleotide sequence of SEQ ID NO:1.

21. The isolated polynucleotide sequence of claim 15, wherein the isolated polynucleotide sequence comprises the polypeptide sequence of SEQ ID NO:2.

22. A method of inducing or enhancing production or cellular secretion of at least one endogenous secondary metabolite by a plant cell, the method comprising:

transforming the plant cell with an expression vector comprising an expression cassette comprising a gene encoding an ABC-transporter, wherein said ABC-transporter comprises a Walker A box, a Walker B box, and a Nucleotide Binding Fold, and functions to transport at least one secondary metabolite in plant cells;

wherein the secondary metabolite is an endogenous metabolic product of the plant cell, and is transported from the cell to the extracellular space;

wherein the amount of secondary metabolite recoverable from the cell is increased;

selecting a transformed plant cell having an induced or enhanced production of at least

one secondary metabolite; and

propagating such selected transformed plant cell.

23. The method according to claim 22 wherein the secondary metabolite is an alkaloid.

24. The method according to claim 22 wherein the ABC-transporter is of plant, fungal, or mammalian origin.

(9) EVIDENCE APPENDIX

(A) Declaration of Alain Goossens. Submitted May 14, 2007.

(B) Theoudoulou et al., Review, Plant ABC transporters, Biochimica et Biophysica Acta, 2000, pp. 79-103, Vol. 1465. Submitted in an IDS mailed September 18, 2003.

(C) Rea *et al.*,. WO 98/21938, published May 28, 1998. Submitted in an IDS mailed September 18, 2003.

(D) Dudler *et al.*, Journal of Biological Chemistry; 25 March 1992, Vol. 267, No. 9, pp. 5582-5588. First cited by the Examiner in Examiner Action mailed August 11, 2006.

(E) Sidler *et al.*, The Plant Cell, 10:1623-1636. First cited by the Examiner in Examiner Action mailed July 31, 2007.

(10) RELATED PROCEEDINGS APPENDIX

NONE.

Respectfully submitted,

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Date: April 2, 2009

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Goossens et al.

Serial No.: 10/666,778

Filed: September 18, 2003

For: THE USE OF GENES ENCODING MEMBRANE TRANSPORTER PUMPS TO STIMULATE THE PRODUCTION OF SECONDARY METABOLITES IN BIOLOGICAL CELLS

Confirmation No.: 8721

Examiner: R. Kallis, Ph.D.

Group Art Unit: 1638

Attorney Docket No.: 2676-6085US

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. ALAIN GOOSSENS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dr. Alain Goossens hereby declares:

1. I am a named inventor on the above-referenced patent application.

2. I am a Principle Investigator at the Flanders Institute for Biotechnology, University of Ghent and an expert in the field of plant secondary metabolite biology. A copy of my curriculum vitae is attached.

3. I understand that in the Office Action mailed February 14, 2007, the Examiner has rejected the claims as being obvious over a combination of Theodoulou (Biochem. Biophys. Acta 1465 79-103) (hereinafter "Theodoulou") and Dudler *et al.* (J. Biol. Chem. 267:9 5582-5588)

(hereinafter "Dudler").

4. Attached hereto, I present data showing that expression of a number of genes, other than ABC transporters, known or thought to be involved in secondary metabolite production have no effect on the production levels of four separate alkaloids in the plant cells tested.

5. The experimental data presented herewith indicate that one of ordinary skill in the art would not reasonably expect success in increasing production or secretion of secondary metabolites through the overexpression of genes known or thought to be involved in secondary metabolite synthesis.

6. The experimental data presented herewith further indicate that one of ordinary skill in the art would find an increase in production or secretion of secondary metabolites through the overexpression of ABC transporters to be unexpected.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 14/5/7

Dr. Alain Goossens

Tag[1] cells[3]	Annotation	Accession	pPMT Induction[2]	Alkaloid levels in transgenic			
				NIC	TAB	BAS	TAL
MAP3	AP2 transcription factor	CQ808982	Yes	o	o	o	o
C330	AP2 transcription factor	CQ808845	Yes	o	o	o	o
C476	MAPK kinase	CQ808961	No	o	o	o	o
T172	Protein phosphatase 2C	CQ809147	No	o	o	o	o
MC307	Putative protein	AJ966362	No	NT			
MC410	Putative protein	AJ966363	No	NT			
MC304	Putative protein	AJ966361	No	o	o	o	o
MT101	GTP-binding-Putative protein	CQ809052	No	o	o	o	o
MT401	Glutathione S-transferase	CQ809143	No	+	o	o	o
C17	Putative protein	AJ966358	No	NT			
C18	RNA-binding protein	AJ966359	No	o	o	o	o
C127	GH3-like protein	CQ808735	No	o	-	o	o
C175	GH3-like protein	CQ808768	No	o	o	o	o
MC204	Putative protein	CQ809012	No	o	o	o	o
C406	Putative RNA-binding protein	AJ966360	No	o	o	o	o
C360	Putative protein	CQ808877	No	o	o	o	o
MAP2	Putative protein	CQ808981	No	NT			

[1] Expression of all of these genes is co-regulated with the expression of genes encoding known nicotine biosynthesis enzymes such as PMT and QPRT. List of genes comes from Table 2 of De Sutter et al. (Plant J. 2005).
[2] Capacity of the genes to induce expression of the known nicotine biosynthesis genes was checked in BY-2 protoplasts (De Sutter et al., Plant J. 2005).
[3] Effect of overexpression of the genes on alkaloid accumulation in transgenic BY-2 cells. NIC, nicotine; TAB, anatabine; BAS, anabasine; TAL, anatalline; +, positive effect; -, negative effect; o, no effect; NT, no transgenic cell lines obtained (Häkkinen et al., submitted for publication).

Tag[1] cells[2]	Annotation	Accession	Alkaloid levels in transgenic			
			NIC	TAB	BAS	TAL
C228	Arginine decarboxylase(ADC)	AF321137	o	-	-	-
C308	Ornithine decarboxylase (ODC)	AF233849	-	-	-	-
MC212	Quinolinate phosphoribosyltransferase (QPRT)	AB038494	-	-	-	-

[1] These genes encode enzymes known to catalyze nicotine biosynthesis and their expression is co-regulated with the expression of the other known gene involved in nicotine biosynthesis (PMT).
[2] Effect of overexpression of the genes on alkaloid accumulation in transgenic BY-2 cells. NIC, nicotine; TAB, anatabine; BAS, anabasine; TAL, anatalline; -, negative effect; o, no effect; (Häkkinen et al., submitted for publication).

1) Transformation of BY2 with plasmids pK7WGD2-ScPDR5-US50 and pKWGD2-ScPDR5-W303 increase anatabine production

BY-2 Strain	Nicotine[1]		Anatabine[1]		
	Medium	Cells	Medium	Cells	% in medium
GUS	0	2.00	0.18	157	0.1
ScPDR5-US50	0	0.88	7.40	207	3.6
ScPDR5-W303	0	2.03	5.12	74	6.9

[1] Alkaloid accumulation (indicated in µg/flask, with 20-ml BY-2 culture per flask) in transformed BY-2 cells measured 72 hours after elicitation with 50 µM methyl jasmonate. Results are the mean of three independent experiments.

2) Transformation of Hyoscyamus muticus hairy roots with pK7WGD2-ScPDR5-US50 improved pseudotropin and cuscohygrin production

H. muticus Strain	Tropine[1]	Pseudotropine[1]	Cuscohygrine[1]	Hysocyamine[1]
Control	18	196	56	5008
ScPDR5-US50	12	364	552	1905

[1] Alkaloid accumulation (indicated in µg/g dry weight) in transformed BY-2 hairy roots. Results are the mean of two or more independent transgenic lines.

3) Transformation of Nicotiana tabacum BY2 hairy roots with pK7WGD2-ScPDR5-US50 and pKWGD2-ScPDR5-W303 increased anatabin production and transformation of Nicotiana tabacum BY2 hairy roots with pKWGD2-ScPDR5-W303 increased nornicotine production

BY-2 Strain	Nicotine[1]	Nornicotine[1]	Anabasine[1]	Anatabine[1]	Anatalline[1]
Control	7125	216	956	197	193
ScPDR5-US50	5439	143	161	907	161
ScPDR5-W303	3993	895	150	957	187

[1] Alkaloid accumulation (indicated in µg/g dry weight) in transformed BY-2 hairy roots. Results are the mean of three or more independent transgenic lines.

Short CV Alain Goossens

Personal data

Name: Alain Goossens
Birth date: 18/01/1971
Nationality: Belgian

Academic training & Career

Ghent University, Belgium

- 1988-1990: Bachelor in Sciences, Biology
- 1990-1992: Master in Sciences, Botany-biotechnology
  - *Undergraduate thesis:* *- Title: "Characterisation of mutations occurring during T-DNA transformation and tissue culture of plant cells."*
  - Promoter: Prof. Dr. Marc Van Montagu, Department of Genetics
- 1992-1998: PhD training in Sciences-biotechnology
  - *PhD thesis:* *- Title: "Molecular characterisation of the gene encoding arcelin 5, a seed storage protein from insect resistant wild common beans (Phaseolus vulgaris)."*
  - Promoter: Prof. Dr. Marc Van Montagu, Department of Genetics

Universidad Politecnica de Valencia, Spain

- 1998-2000: Postdoctoral studies
  - Title: "Identification of novel sodium targets and transporters in yeast by characterisation of suppressors of the salt sensitivity of Na^+-ATPase disruptants."
  - Promoter: Prof. Dr. Ramon Serrano, Instituto de Biología Molecular y Celular de Plantas

Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University

- 2000-present: Group leader/Principle Investigator at Department of Plant Systems Biology, with Prof. Dr. Dirk Inzé.
  - Research theme: Plant secondary metabolism and metabolic engineering.

SCIENTIFIC PUBLICATIONS in SCI JOURNALS

1. **Goossens, A.**, Geremia, R., Bauw, G., Van Montagu, M. & Angenon, G. Isolation and characterization of arcelin 5 proteins and cDNAs. *Eur. J. Biochem.* 225: 787-795 (1994).(SCI 3.164)
2. **Goossens, A.**, Ardiles Diaz, W., De Keyser, A., Van Montagu, M. & Angenon, G. Nucleotide sequence of an *arcelin 5-I* genomic clone from wild *Phaseolus vulgaris* (Accession No. Z50202)(PGR95-075). *Plant Physiol.* 109: 722 (1995). (SCI 6.114)

3. Hamelrijck, T.W., Poortmans, F., **Goossens, A.**, Angenon, G., Wyns, L. & Loris R. Crystallographic structure of arcelin-5, a lectin-like defense protein from *Phaseolus vulgaris*. *J. Biol. Chem.* 271: 32796-32802 (1996). (SCI 5.854)
4. Dillen, W., De Clercq, J., **Goossens, A.**, Van Montagu, M. & Angenon G. *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. *Theor. Appl. Genet.* 94: 151-158 (1997). (SCI 3.063)
5. **Goossens, A.**, Dillen, W., De Clercq, J., Van Montagu, M. & Angenon, G. The *arcelin-5* gene of *Phaseolus vulgaris* directs high seed-specific expression in transgenic *Phaseolus acutifolius* and *Arabidopsis* plants. *Plant Physiol.* 120: 1095-1104 (1999). (SCI 6.114)
6. **Goossens, A.**, Van Montagu, M. & Angenon, G. Co-introduction of an antisense gene for an endogenous seed storage protein can increase expression of a transgene in *Arabidopsis thaliana* seeds. *FEBS Lett.* 456: 160-164 (1999). (SCI 3.415)
7. **Goossens, A.**, Quintero, C., Dillen, W., De Rycke, R., Flower Valor, J., De Clercq, J., Van Montagu, M., Cardona, C. & Angenon, G. Analysis of bruchid resistance in the wild common bean accession G02771: no evidence for insecticidal activity of arcelin 5. *J. Exp. Bot.* 51: 1229-1236 (2000). (SCI 3.336)
8. **Goossens, A.**, de La Fuente, N., Forment, J., Serrano, R. & Portillo, F. Regulation of yeast H+-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. *Mol. Cell. Biol.* 20: 7654-7661 (2000). (SCI 7.093)
9. **Goossens, A.**, Dever, T.E., Pascual-Ahuir, A. & Serrano, R. The protein kinase Gcn2p mediates sodium toxicity in yeast. *J. Biol. Chem.* 276: 30753-30760 (2001). (SCI 5.854)
10. **Goossens, A.**, Forment, J. & Serrano, R. Involvement of Nst1p/YNL091w and Msl1p, a U2B splicing factor, in *Saccharomyces cerevisiae* salt tolerance. Yeast 19: 193-202 (2002). (SCI 2.301)
11. De Jaeger, G., Scheffer, S., Jacobs, A., Zambre, M., Zobell, O., **Goossens, A.**, De Picker, A. & Angenon, G. Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nature Biotechnol.* 20: 1265-1268 (2002). (SCI 22.738)
12. **Goossens, A.**, Häkkinen, S.T., Laakso, I., Oksman-Caldentey, K.M. & Inzé, D. Secretion of secondary metabolites by PDR-type ABC transporters in plant cell suspension cultures. *Plant Physiol.* 131: 1-4 (2003). (SCI 6.114)
13. **Goossens, A.**, Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inzé, D. & Oksman-Caldentey, K.M. A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc. Natl. Acad. Sci. USA* 100: 8595-8600 (2003). (SCI 10.231)
14. Zambre, M., **Goossens, A.**, Cardona, C., Van Montagu, M., Terryn, N. & Angenon G. A reproducible genetic transformation system for cultivated *Phaseolus acutifolius* (tepary bean) and its use to assess the role of arcelins in resistance to the Mexican bean weevil. *Theor. Appl. Genet.* 110: 914-924 (2005). (SCI 3.063)

15. Kwade, Z., Swiatek, A., Azmi, A., **Goossens, A.**, Inzé, D., Van Onckelen, H. & Roef, L. Identification of four adenosine kinase isoforms in tobacco by-2 cells and their putative role in the cell cycle regulated cytokinin metabolism. *J. Biol. Chem.* 280: 17512-17519 (2005). (SCI 5.854)
16. Wolucka, B.A., **Goossens, A.** & Inzé, D. Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* 56: 2527-2538 (2005). (SCI 3.336)
17. De Sutter, V., Vanderhaeghen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Inzé, D., **Goossens, A.** & Hilson, P. Exploration of jasmonate signaling via automated and standardized transient expression assays in tobacco cells. *Plant J.* 44: 1065-1076 (2005). (SCI 6.969)
18. Rischer, H., Oresic, M., Seppänen-Laakso, T., Katajamaa, M., Lammertyn, F., Ardiles-Diaz, W., Van Montagu, M.C.E., Inzé, D., Oksman-Caldentey, K.-M. & **Goossens, A.** Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proc. Natl. Acad. Sci. USA* 103: 5614-5619 (2006). (SCI 10.231)
19. Van Nieuwerburgh, F.C., Van de Casteele, S.R., Maes, L., **Goossens, A.**, Inzé, D., Van Bocxlaer, J. & Deforce, D.L. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. *J. Chromatogr. A* 1118: 180-187 (2006). (SCI 3.096)
20. **Goossens, A.** & Rischer, H. (2006). Implementation of functional genomics for gene discovery in alkaloid producing plants. *Phytochemistry Reviews*, in press (2006).

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(54) Title: GLUTATHIONE–S–CONJUGATE TRANSPORT IN PLANTS

(57) Abstract

The invention includes an isolated DNA encoding a plant GS–*X* pump polypeptide and an isolated preparation of a plant GS–*X* pump polypeptide. Also included is an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS–*X* pump gene. The invention also includes cells, vectors and transgenic plants having an isolated DNA encoding a plant GS–*X* pump and methods of use thereof. In addition, the invention relates to plant GS–*X* pump promoter sequences and the uses thereof.

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GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS

BACKGROUND OF THE INVENTION

Animal and plant cells have the capacity to eliminate a diversity of lipophilic toxins from the cytosol following conjugation of the toxin with glutathione (GSH) (Ishikawa et al., 1997, *Bioscience Reports.* 17:189-208; Martinoia *et al.*, 1993, *Nature* 364:247-249; Li *et al.*, 1995, *Plant Physiol.* 107:1257-1268). This process is mediated by the glutathione *S*-conjugate (GS-*X*) pumps which are novel MgATP-dependent transporters that catalyze the efflux of GS-conjugates and glutathione disulfide (GSSG) from the cytosol via the plasma membrane and/or endomembranes. GS-*X* pumps are considered to constitute a terminal phase of xenobiotic detoxification in animals and plants.

The metabolism and detoxification of xenobiotics comprises three main phases (Ishikawa, 1992, *supra*). Phase I is a preparatory step in which toxins are oxidized, reduced or hydrolyzed to introduce or expose functional groups having an appropriate reactivity. Cytochrome P450 monooxygenases and mixed function oxidases are examples of phase I enzymes. In phase II, the activated derivative is

conjugated with GSH, glucuronic acid or glucose. In the case of the GSH-dependent pathway, *S*-conjugates of GSH are formed by cytosolic glutathione-*S*-transferases (GSTs). In the final phase, phase III, of the GSH-dependent pathway, GS-conjugates are eliminated from the cytosol by the GS-*X* pump.

The GS-*X* pump is unique in its exclusive use of MgATP, rather than preformed transmembrane ion gradients, as a direct energy source for organic solute transport. Although an understanding of the constituents of GS-*X* pumps is relevant to an understanding of the mechanism by which cells combat, for example, chemotherapeutic agents and herbicides, there has until recently been a paucity of information on the molecular identity of GS-*X* pumps, particularly in plants.

A 190 kDa membrane glycoprotein encoded by the human multidrug resistance-associated protein gene (*MRP1*) has been implicated in the resistance of small cell lung cancer cell lines to a number of chemotherapeutic drugs (Cole *et al.*, 1992, *Science* 258:1650-1654). This glycoprotein catalyzes the MgATP-dependent transport of leukotriene C_4 and related glutathione-*S*-conjugates (Leier *et al.*, 1994, *J. Biol. Chem.* 269:27807-27810; Muller *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:13033-13037; Zamam *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92:7690-7694).

MRP1 is a member of the ATP binding cassette (ABC) superfamily of transporter proteins. Distributed throughout the major taxa, ABC transporters catalyze the MgATP-dependent transport of peptides, sugars, ions and lipophiles across membranes. ABC transporters comprise one or two copies each of two basic structural elements, a hydrophobic integral membrane sector containing approximately six transmembrane α helices and a cytoplasmically oriented ATP-binding domain known as a nucleotide binding fold (NBF) (Hyde *et al.*, 1990, *Nature* 346:362-365; Higgins, 1995, *Cell* 82:693-696). The NBFs are a diagnostic feature of ABC transporters and are 30% identical between family members over a span of about 200 amino acid residues, having two regions known as a Walker A and a Walker B box (Walker *et al.*, 1992, *EMBO J.* 1:945-951), and also having an ABC signature motif (Higgins, 1995, *supra*).

ABC family members in eukaryotes include mammalian P-glycoproteins (P-gps or MDRs), some of which are implicated in drug resistance and others in lipid translocation (Ruetz *et al.*, 1994, *Cell* 77:1071-1081), the pleiotropic drug resistance protein (PDR5) and STE6 peptide mating pheromone transporter of yeast, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, the malarial *Plasmodium falciparum* chloroquine transporter (PFMDR1) and the major histocompatibility (MHC) transporters responsible for peptide translocation and antigen presentation (Balzi *et al.*, 1994, *J. Bioenerg. Biomemb.* 27:71-76; Higgins, 1995, *supra*).

Sequence comparisons between MRP1 and other ABC transporters reveal two major subgroups among these proteins (Cole *et al.*, 1992, *supra*; Szczypka *et al.*, 1994, *J. Biol. Chem.* 269:22853-22857). One subgroup comprises MRP1, the *Saccharomyces cerevisiae* cadmium factor (*YCF1*) gene, the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA) and the CFTRs. The other subgroup comprises the multiple drug resistance proteins (MDRs), MHC transporters and STE6.

The invention described herein relates to bioremediation (specifically phytoremediation), plant responses to herbicides, plant-pathogen interactions and plant pigmentation.

With respect to bioremediation, the massive global expansion in industrial and mining activities during the last two decades together with changes in agricultural practices, has markedly increased contamination of groundwaters and soils with heavy metals. Indeed, it is estimated that the annual toxicity of metal emissions exceeds that of organics and radionuclides combined (Nriagu *et al.*, 1988, *Nature* 333:1340138). Since soil and water contamination results in the uptake of heavy metals and toxins by crop plants, and eventually humans, there remains a need for a means of manipulating the ability of a plant to sequester compounds from the soil in order to better manage soil detoxification through bioremediation using native species or genetically engineered organisms.

Regarding herbicides, these compounds are generally low molecular weight, lipophilic compounds that readily penetrate cells in a passive manner. Having entered cells, herbicides inhibit plant-specific processes such as photosynthetic electron transport (*e.g.*, atrazine, chlortoluron) or the biosynthesis of essential amino acids (*e.g.*, glyphosate, chlorsulfuron or phosphotricine), porphyrins (*e.g.*, acidofluorfen), carotenoids (*e.g.*, norflurazon), fatty acids (*e.g.*, diclofop) or cellulose (*e.g.*, dichlobenil) (Boger *et al.*, 1989, *Target Sites of Herbicide Action*, CRC Press, Boca Raton, FL; Devine *et al.*, 1993, *Physiology of Herbicide Action*, Prentice Hall, Englewood Cliffs, NJ). Plants that are naturally tolerant of certain herbicides either contain a cellular target that does not interact with the herbicide, have efficient systems for inactivation of the herbicide, or have a high capacity for excluding or eliminating the herbicide from the target.

Herbicide metabolism comprises the three phases described above for general xenobiotic metabolism. The first two phases (the first being oxidation and hydrolysis and the second being conjugation with GSH or glucose) contribute to detoxification by decreasing the intrinsic biochemical activity of the herbicide and/or by increasing its hydrophilicity. These two phases render the herbicide less mobile in the plant. The third phase (compartmentation) is often critical for sustained detoxification since the conjugates themselves may interfere with metabolism. For example, the herbicide synergist tridiphane, is converted to its corresponding GS-conjugate in plants to generate a potent inhibitor of atrazine metabolism. (Lamoureux *et al.*, 1986, *Pestic. Biochem. Physiol.* **26**:323-342).

Likewise, and more generally, GS-conjugates of any given herbicide would be expected to act as end-product inhibitors of GSTs and thereby impair long-term detoxification unless they are removed from the intracellular compartment, usually the cytosol, in which they are formed. Since the vacuolar GS-*X* pump of plants is known to transport several GS-herbicide conjugates, for example, those of the chloroacetanilide herbicides (metolachlor) and triazines (simetryn) (Martinoia *et al.*, 1993, *supra*; Li *et al.*, 1995, *supra*), there is a long felt need for a knowledge of the

molecular identity of this transporter or family of transporters. Such knowledge will enable the development of new strategies for increasing or decreasing the resistance of plants to herbicides.

With regard to plant-pathogen interactions, a key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. The majority of the research on plant-pathogen interactions has centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, the discovery of the molecular mechanism by which these compounds are sequestered within a plant is crucial to the development of plants with increased pathogen resistance.

With regard to plant pigmentation, functional analyses of the maize gene, *Bronze-2*, which participates in anthocyanin pigment biosynthesis, suggest that one of the endogenous substrates for the plant vacuolar GS-*X* pump are anthocyanin-GS conjugates (Marrs *et al.*, 1995, *Nature*, 375:397-400). Anthocyanins share a common biosynthetic origin and core structure based on cyanidin-3-glucoside. It is through the species-specific decoration of cyanidin-3-glucoside by hydroxylation, methylation, glucosylation and acylation that the wide spectrum of red, blue and purple colors in the vacuoles of flowers, fruits and leaves is produced. The molecular nature of the plant GS-*X* pump which mediates transport of anthocyanin-GS conjugates was not known in the art until the present invention. There remains a need to determine the molecular nature of the GS-*X* pump responsible for transport of anthocyanin-GS conjugates in order that plant coloration may be manipulated at the molecular level.

The present invention satisfies the aforementioned needs.

BRIEF SUMMARY OF THE INVENTION

The invention includes an isolated DNA encoding a plant GS-*X* pump polypeptide. In one aspect, the isolated DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.

The invention also includes an isolated preparation of a polypeptide comprising a plant GS-*X* pump. In one aspect of this aspect of the invention, the polypeptide is selected from the group consisting of AtMRP1, AtMRP2, and any mutants, derivatives, homologs and fragments thereof having GS-*X* pump activity.

Also included in the invention is a recombinant cell comprising an isolated DNA encoding a plant GS-*X* pump polypeptide. In one aspect, the cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

Further included in the invention is a vector comprising an isolated DNA encoding a plant GS-*X* pump polypeptide.

The invention also includes an antibody specific for a plant GS-*X* pump polypeptide.

In addition, an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-*X* pump gene is included in the invention and a cell and a vector comprising this isolated preparation of a nucleic acid are further included.

The invention also relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-*X* pump.

In addition, the invention relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-*X* pump gene.

Further, there is included a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.

The invention further relates to an isolated DNA comprising a plant GS-X pump promoter sequence. In one aspect, the promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.

Also included in this aspect of the invention is a cell and a vector comprising an isolated DNA comprising a plant GS-*X* plant promoter sequence.

The invention additionally relates to a transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising a GS-*X* pump promoter sequence, wherein the GS-*X* pump promoter sequence is selected from the group consisting of an *AtMRP1*, an *AtMRP2* and a *YCF1* promoter sequence. The promoter sequence may also have operably fused thereto a reporter gene.

There is also included in the invention a method of identifying a compound capable of affecting the expression of a plant GS-*X* gene. The method comprises providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto, adding to the cell a test compound, and measuring the level of reporter gene activity in the cell, wherein a higher or a lower level of reporter gene activity in the cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that the test compound is capable of affecting the expression of a plant GS-*X* pump gene.

In addition, the invention relates to a method of removing xenobiotic toxins from soil. The method comprises growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-*X* pump.

Also included is a method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-*X* pump.

The invention further relates to a method of generating a transgenic pathogen resistant plant comprising introducing to the cells of the plant an isolated DNA encoding a GS-*X* pump, wherein the pump is capable of transporting glutathionated isoflavonoid alexins into the cells of the plant.

Additionally, there is included a method of manipulating plant pigmentation comprising modulating the expression of a GS-*X* pump protein in the plant, wherein the GS-*X* pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

The invention also relates to a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-*X* pump, wherein the DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

Further included is a method of manipulating the expression of a gene in a plant cell. The method comprises operably fusing a GS-*X* pump promoter sequence to the DNA sequence encoding the gene to form a chimeric DNA, and generating a transgenic plant, the cells of which comprise the chimeric DNA, wherein upon activation of the GS-*X* pump promoter sequence, the expression of the gene is manipulated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of graphs depicting differential sensitivities of DYT165 cells (wild type, **Figure 1A**) and DTY167 cells (*ycf1*Δ mutant, **Figure 1B**) to growth inhibition by 1-chloro-2,4-dinitrobenzene (CDNB). Cells were grown at 30°C for 24 hours to an $OD_{600\ nm}$ of approximately 1.4 in YPD medium before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60 μM CDNB. $OD_{600\ nm}$ was measured at the times indicated.

Figure 2 is a graph depicting the time course of [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in the absence (-MgATP) or presence of 3 mM MgATP (+MgATP) in reaction media containing 66.2 μM [^{3}H]DNP-GS, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol, and 25 mM Tris-MES (pH 8.0) at 25°C. Values shown are means ± S.E. (n = 3).

Figure 3 is a series of graphs depicting the kinetics of uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. **Figure 3A:** MgATP concentration-dependence of uncoupler-insensitive uptake. **Figure 3B:** DNP-GS concentration-dependence of MgATP-dependent uncoupler-insensitive uptake. The MgATP concentration-dependence of uptake was measured with 66.2 μM [^{3}H] DNP-GS. The DNP-GS concentration-dependence of uptake was measured with 3 mM MgATP. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5 μM gramicidin D. The kinetic parameters for vacuolar membrane vesicles purified from DTY165 cells were $K_{m(MgATP)}$ 86.5 ± 29.5 μM, $K_{m(DNP\text{-}GS)}$ 14.1 ± 7.4 μM, $V_{max(MgATP)}$ 38.4 ± 5.6 nmol/mg/10 minutes, $V_{max(DNP\text{-}GS)}$ 51.0 ± 6.3 nmol/mg/10 minutes. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *J. Soc. Ind. Appl. Math.* 11:431-441). Values shown are means ± S.E. (n = 3).

Figure 4 is a series of graphs depicting sucrose density gradient fractionation of vacuolar membrane-enriched vesicles prepared from DYT165 cells. One ml (1.1 mg protein) of partially purified vacuolar membrane vesicles derived from vacuoles prepared by the Ficoll flotation technique were applied to a linear sucrose density gradient (10-40%, w/v) and analyzed for protein (**Figure 4A**), α-mannosidase activity (**Figure 4B**), V-ATPase activity (**Figure 4C**), and MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake (**Figure 4D**). [^{3}H]DNP-GS uptake and enzyme activity were assayed as described herein in Table 4 and the accompanying text.

Figure 5 includes a graph (**Figure 5A**) depicting the effect of transformation with pYCF1-HA or pRS424 on MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membranes purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 66.2 μM [^{3}H]DNP-GS and 5 μM gramicidin D. Also shown (**Figure 5B**) is an image of a gel depicting immunoreaction of vacuolar membrane proteins prepared from pYCF1-HA-

transformed and pRS424-transformed DTY165 and DTY167 cells with mouse monoclonal antibody raised against the 12CA5 epitope of human influenza hemagglutinin. All lanes were loaded with 25 μg of delipidated membrane protein and subjected to SDS-polyacrylamide gel electrophoresis and Western analysis as described herein. The M_r of YCF1-HA (boldface type) and the positions of the M_r standards are indicated on the figure.

Figure 6 is a series of graphs depicting transformation with pYCF1-HA (**Figure 6A**) or pRS424 (**Figure 6B**) on the sensitivity of DTY167 cells to growth retardation by CDNB. Cells were grown at 30°C for 24 hours to an $OD_{600\ nm}$ of approximately 1.4 in AHC medium (Kim *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* **91**:6128-6132) before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60 μM CDNB. $OD_{600\ nm}$ was measured at the times indicated.

Figure 7 is a series of photomicrographs of DTY165 (**Figures 7A** and **7C**) and DTY167 cells (**Figures 7B** and **7D**) after incubation with monochlorobimane. Cells were grown in YPD medium for 24 hours at 30°C and 100 μl aliquots of cell suspensions were transferred into 15 ml volumes of fresh YPD medium containing 100 μM monochlorobimane. After incubation for 6 hours, the cells were washed and examined in fluorescence (**Figures 7C** and **7D**) or Nomarski mode (**Figures 7A** and **7B**) as described herein.

Figure 8 is a series of graphs depicting uptake of Cd^{2+} into vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake of $^{109}Cd^{2+}$ by DTY165 membranes (**Figure 8A**) or DTY167 membranes (**Figure 8B**) was measured in the absence of MgATP plus (O) or minus GSH (1 mM) (□) or in the presence of MgATP (3 mM) plus (●) or minus (■) GSH. $^{109}Cd_2SO_4$ and gramicidin-D were added at concentrations of 80 μM and 5 μM, respectively. **Figure 8C**: Rate of $^{109}Cd^{2+}$ uptake by DTY165 membranes plotted as a function of the total concentration of Cd^{2+} ($[Cd^{2+}]_{total}$) added to uptake medium containing 1 mM GSH, 3 mM MgATP and 5 μM gramidicin-D. Values shown are means ± SE (n = 3-6).

Figure 9 is a series of graphs depicting purification of cadmium glutathione complexes by gel-filtration (**Figure 9A**) and anion-exchange chromatography (**Figures 9B** and **9C**). Twenty mM $^{109}Cd_2SO_4$ was incubated with 40 mM GSH at 45°C for 24 hours and the mixture was chromatographed on Sephadex G-15 to resolve a high molecular weight ^{109}Cd-labeled component (*HMW-^{109}Cd.GS*) from a low molecular weight component (*LMW-^{109}Cd.GS*) (**Figure 9A**). The peaks corresponding to *HMW-^{109}Cd.GS* and *LMW-^{109}Cd.GS* were then chromatographed on Mono-Q and eluted with a linear NaCl gradient (---) (**Figure 9B** and **9C**). ^{109}Cd (cpm x 10^{-3}) was determined on 5 µl aliquots of the column fractions by liquid scintillation counting.

Figure 10 is a series of graphs depicting the kinetics of MgATP-dependent, uncoupler-insensitive $^{109}Cd.GS_2$ (*HMW-^{109}Cd.GS*, **Figure 10A**) and ^{109}Cd.GS (*LMW-^{109}Cd.GS*, **Figure 10B**) uptake. DNP-GS was added at the concentrations (µM) indicated to DTY165 membranes (●,○,■,□,Δ) or DTY167 membranes (◊). A secondary plot of the apparent Michaelis constants for $Cd.GS_2$ uptake (K_m^{app}/$Cd.GS_2$) as a function of DNP-GS concentration is shown (**Figure 10C**). The kinetic parameters for $Cd.GS_2$ transport by DTY165 membranes were K_m, 39.1 ± 14.1 µM, V_{max}, 157.2 ± 30.4 nmol/mg/10 minutes and $K_{i(DNP\text{-}GS)}$, 11.3 ± 2.1 µM. Kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *supra*). Values shown are means ± SE (n = 6).

Figure 11 is a graph depicting matrix-assisted laser desorption mass spectrometry (MALD-MS) of *HMW-Cd.GS*. MALD-MS was performed on Sephadex G-15-, Mono-Q-purified *HMW-Cd.GS* as described herein. The molecular structure inferred from a mean m/z ratio of 725.4 ± 0.7 (n = 9) and average Cd.GS stoichiometry of 0.5 [*bis*(glutathionato)cadmium, $Cd.GS_2$, molecular weight 724.6 Da] is shown.

Figure 12 is an image of a gel depicting induction of *YCF1* expression and YCF1-dependent $Cd.GS_2$ and DNP-GS transport by pretreatment of DTY165 cells with $CdSO_4$ (Cd^{2+}, 200 µM) or CDNB (150 µM) for 24 hours. *YCF1*-specific mRNA and 18S rRNA were detected in the total RNA extracted from control or pretreated

cells (10 μg/lane) by RNase protection. Uptake of $^{109}Cd.GS_2$ (50 μM) or [^{3}H]DNP-GS (66.2 μM) by vacuolar membrane vesicles was measured in standard uptake medium containing 5 μM gramicidin-D. Values shown are means ± SE (n = 3).

Figure 13A and **13B** is the sequence of *AtMRP2* cDNA (SEQ ID NO:1). Lower case letters correspond to 5'- and 3'-untranslated regions (UTRs).

Figure 14A-D is the genomic sequence of *AtMPR2* (SEQ ID NO:2). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

Figure 15 is the deduced amino acid sequence of AtMRP2 (SEQ ID NO:3).

Figure 16A and **16B** is the sequence of *AtMRP1* cDNA (SEQ ID NO:4). Lower case letters correspond to 5'- and 3'-UTRs.

Figure 17A-D is the genomic sequence of *AtMRP1* (SEQ ID NO:5). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

Figure 18 is the deduced amino acid sequence of AtMRP1 (SEQ ID NO:6).

Figure 19 is a series of graphs depicting the time course and concentration-dependence of DNP-GS uptake in *AtMRP1*-transformed yeast. **Figure 19A** is a graph depicting the time course of [^{3}H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed or pYES3-transformed DTY168 cells. MgATP-dependent uptake was measured in reaction media containing 61.3 μM [^{3}H]DNP-GS, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown are means ± SE (n = 3). **Figure 19B** is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive uptake of [^{3}H]DNP-GS by membrane vesicles purified from pYES3-*AtMRP1*-

transformed DTY168 cells. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5 μM gramicidin-D. The kinetic parameters for uptake were $K_{m(DNP\text{-}GS)}$ 49.7 ± 15.4 μM, V_{max} 6.0 ± 1.7 nmol/mg/10 minutes. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, supra). Values shown are means ± SE (n = 3).

Figure 20 is a series of graphs depicting sensitivity of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed DTY168 cells. Uptake was measured for 10 minutes in standard uptake medium containing the indicated concentrations of vanadate. In **Figure 20A**, there is a graph depicting total MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES-transformed DTY168 cells. In **Figure 20B**, there is a graph depicting AtMRP1-dependent uptake. I_{50} (exclusive of uninhibitable AtMRP1-independent component) = 8.3 ± 3.2 μM. Values shown are means ± SE (n = 3).

Figure 21 is a series of graphs depicting the hydropathy alignment of AtMRP2, AtMRP1, *S. cerevisiae* YCF1 (ScYCF1), human MRP1 (HmMRP1) and rat cMOAT (RtCMOAT).

Figure 22 is a diagram depicting domain comparisons between AtMRP1, ScYCF1, HmMRP1, RtCMOAT, rabbit EBCR (RbEBCR) and HmCFTR. The domains indicated are the N-terminal extension (NH_2), first and second sets of transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH).

Figure 23 is the promoter sequence of the *Arabidopsis AtMRP1* gene (SEQ ID NO:7). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

Figure 24 is the promoter sequence of the *Arabidopsis AtMRP2* gene (SEQ ID NO:8). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

Figure 25 is a graph depicting MgATP-dependence of [^{3}H]medicarpin uptake by vacuolar membrane vesicles before (Medicarpin/GSH) and after maize GST-mediated conjugation with GSH (Medicarpin-GS). [^{3}H]medicarpin or [^{3}H]medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Values shown are means ± SE (n = 3).

Figure 26 is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive [^{3}H]medicarpin-GS uptake into vacuolar membrane vesicles. Uptake was allowed to proceed for 20 minutes in standard uptake medium containing 3 mM MgATP and 5 μM gramicidin D. The kinetic parameters were K_m 21.5 ± 15.5 μM and V_{max} 77.8 ± 23.3 nmol/mg/20 minutes. Values shown are means ± SE (n = 3).

Figure 27 is a series of graphs depicting concentration-dependence of MgATP-dependent, uncoupler-insensitive C_3G-GS, IAA-GS and ABA-GS uptake by vacuolar membrane vesicles purified from *V. radiata* (**Figure 27A**) and *Z. mays* (**Figure 27B**). Uptake was allowed to proceed for 10 minutes in reaction medium containing 50 μM GS-conjugate, 400 mM sorbitol, 3 mM MgATP, 50 mM KCl, 0.1% (w/v) BSA, 5 μM gramicidin-D and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown are means ± SE (n = 3).

Figure 28 is an image of a photograph depicting the growth of wild type (WT) and *YCF1* transgenic *Arabidopsis* (YCF1) seeds on media containing $CdSO_4$ (200 μM) or 1-chloro-2,4-dinitrobenzene (CDNB, 25 μM). Transgenic plants were generated as described herein.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based upon the molecular identification of a new class of membrane transporter in yeast and plants, the GS-*X* pump. As a result of the present

invention, new insights into the membrane transport phenomena associated with heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation are evident. These insights provide a means, as is evident from the description of the present invention, for the manipulation of plants and the cells thereof, to affect heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation.

The process of "storage excretion" is a necessity for plants. Whereas mammals have the option of excreting GS-conjugates to the extracellular medium for elimination by the kidneys, plants are nearly totally reliant on the sequestration of noxious compounds in the central vacuole, which frequently accounts for 40-90% of total intracellular volume. Due to the virtual absence of specialized excretory organs and the presence of massive vacuoles in plants, a process (intracellular compartmentation) that is probably only an intermediate step in the elimination of xenobiotics from the cytosol of mammalian cells, is believed to constitute a terminal phase of detoxification in plants.

The data which are described herein establish that the yeast gene *YCF1* and two plant homologs of *YCF1*, *AtMRP1* and *AtMRP2*, isolated from *Arabidopsis thaliana*, each encode a vacuolar GS-*X* pump. The data further establish that the GS-*X* pump participates in herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions (exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins).

The plant *AtMRP1* and *AtMRP2* gene products use MgATP as an energy source for the transport of glutathionated derivatives of both endogenous and exogenous compounds in plants and thus, the discovery of these genes in the present invention is important at three levels. The identification of these genes and their encoded products represents the first identification of ABC transporters in plants for

which a biochemical function is defined. The discovery establishes, contrary to the prevailing chemiosmotic model for solute transport in plants, that many energy-dependent solute transport processes in plants are not driven by a transmembrane H+ electrochemical potential difference. Further, the identification and isolation of these genes and their encoded products permits a plant element, critical for removal of compounds from the cytosol that can form glutathionine *S*-conjugates, to be manipulated.

It has been discovered in the present invention that two plant genes, *AtMRP1* and *AtMRP2*, are the structural and functional homologs of the gene encoding yeast YCF1. Proteins encoded by plant *AtMRP1* and *AtMRP2* thus represent a new subclass of ATP binding cassette transporters.

It has been further discovered in the present invention that the yeast YCF1 protein, a GS-*X* pump, is capable of MgATP-energized transport of organic GS-conjugates and of MgATP-energized transport of cadmium upon complexation with GSH. In addition, when plants have introduced into the cells thereof the *YCF1* gene (a transgenic plant comprising *YCF1*), expression of *YCF1* therein confers upon the plants resistance to both inorganic and organiC xenobiotics exemplified by cadmium and 1-chloro-2,4-dinitrobenzene, respectively.

Also discovered in the present invention is the fact that *AtMRP1* and *AtMRP2*, when expressed in a strain of yeast which is deficient in YCF1, can substitute for *YCF1* as a GS-*X* pump. In addition, transformation of plants by *YCF1* confers upon the plant properties which are characteristic of *YCF1* gene expression. Thus, it appears that *YCF1* and the *AtMRP* genes are essentially functionally interchangeable.

In addition, there is provided as part of the invention the promoter/regulatory sequences which control expression of the plant *AtMRP1* and *AtMRP2* genes of the invention. These promoter sequences are useful for the identification of compounds which affect expression of thEse genes in plants and for conferring on other genes the ability to respond to factors that modulate *AtMRP1* and/or *AtMRP2* expression.

Further discovered in the present invention is the fact that the plant GS-*X* pump serves to facilitate the vacuolar storage of antimicrobial compounds induced following the hypersensitive response to fungal pathogens in the healthy cells surrounding fungally-induced lesions. Such a process is believed to limit the spread of tissue damage by limiting propagation of the pathogen and spatially delimiting the toxic action of the phytoalexin itself.

Ascription of specific enzymic and regulatory roles to most of the genes of the anthocyanin biosynthetic pathway has been achieved by genetic and biochemical studies of maize with one notable exception, the *Bronze-2* gene. It is known that the characteristic coloration of *Bronze-2 (bz2)* mutants is a consequence of the accumulation of cyanidin-3-glucoside in the cytosol. However, in wild type (*Bz2*) plants, anthocyanins are transported into the vacuole and become purple or red. In the mutant (*bz2*) plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a brown ("bronze") pigment. The biochemical basis for the accumulation of anthocyanins in the cytosol is not known. However, Marrs *et al.*, (1995, *supra*) have discovered that *Bz2* encodes a glutathione *S*-transferase which is responsible for conjugating anthocyanin with GSH. It has now been discovered in the present invention that the plant GS-*X* pump is the entity responsible for the delivery of glutathionated anthocyanins into the vacuole.

Identification of the GS-*X* pump at the molecular level has served to confirm its wide distribution and demonstrate that these transporters constitute a multigene family within the ABC transporter superfamily. The critical finding was that overexpression of the human multidrug resistance-associated protein (*MRP1*) gene (Cole *et al.*, 1992, *supra*) confers increased MgATP-dependent GS-conjugate transport (Muller *et al.*, 1994 *supra*; Leier *et al.*,, 1994, J. Biol. Chem. 269:27807-27810).

Several other closely related GS-*X* pump genes have been characterized. For example, a liver-specific GS-*X* pump (*cMOAT*), mutation of which is believed to cause hereditary hyperbilirubinemia, has been cloned (Paulusma *et al.*, 1996,Science 271:1126-1128). The present invention establishes that YCF1 is a GS-*X* pump. In

addition, as will become apparent from a reading of the present description, two plant genes, *AtMRP1* and *AtMRP2* have been discovered in the present invention to encode homologs of MRP1, YCF1 and cMOAT.

The identification of YCF1 as a vacuolar GS-*X* pump is described in detail in the experimental details section. Similarly, the identification of two plant homologs of YCF1, AtMRP1 and AtMRP2, is also described in detail in the experimental details section. Once armed with the present invention, the skilled artisan will know how to identify and isolate genes encoding other plant GS-*X* pumps involved in sequestration of a variety of compounds in plants by following the procedures described herein.

A plant gene encoding a GS-*X* pump is isolated using any one of several known molecular procedures. For example, primers comprising conserved regions of the sequences of any of *YCF1*, *AtMRP1* or *AtMRP2*, or in fact primers comprising conserved regions of any MRP subclass (*i.e.*, probes directed to human *MRP1 cMOAT*, and other MRP genes) may be used as probes to isolate, by polymerase chain reaction (PCR) or by direct hybridization, as yet unknown *YCF1*, *AtMRP1* or *AtMRP2* homologs in a DNA library comprising specific plant DNAs. Alternatively, antibodies directed against YCF1, AtMRP1 or AtMRP2 may be used to isolate clones encoding a GS-*X* pump from an expression library comprising specific plant DNAs. The isolation of primers, probes, molecular cloning and the generation of antibodies are procedures that are well known in the art and are described, for example, in Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York) and in Harlow *et al.* (1988, Antibodies, A Laboratory Manual, Cold Spring Harbor, New York).

The invention includes an isolated DNA encoding a plant GS-*X* pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell. Preferably, the DNA encoding a plant GS-*X* pump is at least about 40% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-*X* pump is at least

about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-*X* pump is *Arabidopsis AtMRP1* or *AtMRP2*. Most preferably, the isolated DNA encoding a plant GS-*X* pump is SEQ ID NOS:1, 2, 4 or 5.

Thus, the invention should be construed to include genes which encode *Arabidopsis* AtMRP1 and AtMRP2 and *Arabidopsis AtMRP1* and *AtMRP2*-related genes.

By "GS-*X* pump" as used herein, is meant a protein which transports a glutathione-conjugated compound across a biological membrane.

By the term "DNA encoding a GS-*X* pump" as used herein is meant a gene encoding a polypeptide capable of transporting a glutathionated compound across a biological membrane.

By "*AtMRP*-related gene" as used herein, is meant a gene encoding a GS-*X* pump which is a member of the *MRP/YCF1/cMOAT* family of genes. An *AtMRP1* or *AtMRP2*-related gene may be present in a cell which also encodes an *AtMRP* gene or it may be present in a different cell and in a different plant species.

As described in the Experimental Detail section, *AtMRP* genes encode proteins which have specific domains located therein, namely, the N-terminal extension, transmembrane spans, TM1 and TM2, nucleotide binding folds, NBF1 and NBF2, putative CFTR-like regulatory domain (R) and the C-terminus. An *AtMRP*-related gene is therefore also one in which selected domains in the related protein share significant homology (at least about 40% homology) with the same domains in either of YCF1, AtMRP1 or AtMRP2. For example, when the R-domain in the AtMRP-related protein shares at least about 40% homology with the R domain in YCF1, AtMRP1 or AtMRP2, and when the product of that is a GS-*X* pump, then that gene is an *AtMRP*-related gene. Similarily, when the N-terminal extension in the AtMRP-related protein shares at least about 40% homology with the N-terminal extension in

YCF1, AtMRP1 or AtMRP2, and when the product of that is a GS-*X* pump, then that gene is an *AtMRP*-related gene. It will be appreciated that the definition of an *AtMRP*-related gene encompasses those genes having at least about 40% homology in any of the described domains contained therein with the same or a similar domain in either of YCF1, AtMRP1 or AtMRP2. In addition, when the term homology is used herein to refer to the domains of these proteins, it should be construed to be applied to homology at both the nucleic acid and the amino acid levels.

While a significant homology between similar domains in *AtMRP*-related genes or their protein products is considered to be at least about 40%, preferably, the homology between domains is at least about 50%, more preferably, at least about 60%, even more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% and most preferably, the homology between similar domains is about 99% between a domain in an *AtMRP*-related gene or protein product thereof, and at least one of *YCF1*, *AtMRP1* or *AtMRP2* or the protein products thereof.

Plants from which *AtMRP1*, *AtMRP2* or *YCF1* related genes may be isolated include any plant in which the GS-*X* pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

By the term "glutathionated-conjugated compound" as used herein is meant a compound, *e.g.*, a metal, a xenobiotic, a isoflavonoid phytoalexin, anthocyanin or auxin, which is chemically conjugated to glutathionine. Conjugation of compounds to glutathione occurs naturally within cells and organisms and may also be accomplished enzymatically or non-enzymatically *in vitro* as described herein in the experimental details section.

Also included in the invention is an isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump. Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump is at least about 40% homologous to a biologically active polypeptide fragment

of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90%, and even more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump is about 200 nucleotides in length. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X* pump is about 400 nucleotides, even more preferably, at least about 600, yet more preferably, at least about 800, even more preferably, at least about 1000, and more preferably, at least about 1200 nucleotides in length.

The invention further includes a vector comprising a gene encoding a plant GS-*X* pump and a vector comprising nucleic acid sequence encoding a biologically active fragment thereof. The procedures for the generation of a vector encoding a plant GS-*X* pump, or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (*e.g.*, pBIN19) containing the target gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Lagrimini *et al.*, 1990, Plant Cell 2:7-18) or its endogenous promoter (Bevan, 1984, Nucl. Acids Res. 12:8711-8721).

Also included in the invention is a cell comprising an isolated DNA encoding a plant GS-*X* pump and a cell comprising an isolated DNA encoding a biologically active fragment thereof. Such a cell is referred to herein as a "recombinant cell."

The procedures for the generation of a cell encoding a plant GS-*X* pump or fragment thereof, are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable cells include, but are not limited to, yeast cells, bacterial cells, mammalian cells, and baculovirus-infected insect cells transformed with the gene for the express purpose of generating GS-*X* polypeptide. In addition, plant cells transformed with the gene for the purpose of producing cells and regenerated plants having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration.

The invention also includes an isolated preparation of a polypeptide comprising a plant GS-*X* pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the isolated preparation of a polypeptide comprising a plant GS-*X* pump is at least about 30% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a polypeptide comprising a plant GS-*X* pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least about 60%, even more preferably, at least about 70%, more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a polypeptide comprising a plant GS-*X* pump is *Arabidopsis* AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a polypeptide comprising a plant GS-*X* pump is SEQ ID NOS: 3 or 6.

Also included in the invention is an isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump. Preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump is at least about 30% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least

about 60%, even more preferably, at least about 70% and yet more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the polypeptide in the isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump is about 60 amino acids in length. More preferably, the polypeptide in the isolated preparation of a biologically active polypeptide fragment of a plant GS-*X* pump is about 130 amino acids, even more preferably, at least about 200, yet more preferably, at least about 300, even more preferably, at least about 350, and more preferably, at least about 400 amino acids in length.

As used herein, the term "homologous" refers to the subunit sequence similarity between two polymeric molecules *e.g.*, between two nucleic acid molecules, *e.g.*, between two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two polypeptide molecules is occupied by phenylalanine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, 5 positions in a polymer 10 subunits in length) of the positions in two polypeptide sequences are homologous then the two sequences are 50% homologous; if 70% of the positions, *e.g.*, 7 out of 10, are matched or homologous, the two sequences share 70% homology. By way of example, the polypeptide sequences ACDEFG and ACDHIK (SEQ ID NOS:9 and 10, respectively) share 50% homology and the nucleotide sequences CAATCG and CAAGAC share 50% homology.

An "isolated DNA," as used herein, refers to a DNA sequence which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally

adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

As used herein, the term "isolated preparation of a polypeptide" describes a polypeptide which has been separated from components which naturally accompany it. Typically, a polypeptide is isolated when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, even more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) of a sample is the polypeptide of interest. The degree of isolation of the polypeptide can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis. For example, a polypeptide is isolated when it is essentially free of naturally associated components or when it is separated from the native compounds which accompany it in its natural state.

As used herein, by the term "biologically active" as it refers to GS-X pump activity as used herein, is meant a polypeptide, or a fragment thereof, which is capable of transporting a glutathionated compound across a biological membrane.

In summary, the invention should be construed to include DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof, which encode GS-*X* pump biological activity.

The invention further features an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS-*X* pump gene, wherein the nucleic acid is capable of inhibiting expression of the GS-*X* pump gene when introduced into cells comprising the GS-*X* pump gene. The nucleic acid is antisense to either a portion or all of a plant GS-*X* pump gene, which gene is preferably *Arabidopsis AtMRP1, Arabidopsis AtMRP2* or a homolog thereof. The "isolated preparation of a nucleic acid" and the "portion" of the gene to which the nucleic acid is antisense, should be of a sufficient length so as to inhibit expression of the desired target gene. The actual length of the isolated preparation of the nucleic acid may vary, and will depend on the particular target gene and the region of that gene which is targetted. Typically, the isolated preparation of the nucleic acid will be at least about 15 contiguous nucleotides; more typically, it will be between about 15 and about 50 contiguous nucleotides, or it may even be more than 50 contiguous nucleotides in length.

As used herein, a sequence of a nucleic acid is "antisense" to a portion or all of a GS-*X* pump gene when the sequence of nucleic acid does not encode a GS-*X* polypeptide. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the GS-*X* pump gene and thus, does not encode a GS-*X* pump polypeptide.

"Complementary," as used herein, refers to the subunit sequence complementarity between two nucleic acids, *e.g.*, two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (*e.g.*, A:T and G:C nucleotide pairs).

In yet another aspect of the invention, there is provided an antibody directed against a plant GS-*X* pump, preferably AtMRP1 or AtMRP2, which antibody

is specific for the whole molecule or either the N-terminal or the C-terminal or internal portions of AtMRP1 or AtMRP2. Methods of generating such antibodies are well known in the art and are described, for example, in Harlow *et al.* (*supra*).

The present invention also provides for analogs of proteins or peptides encoded by *AtMRP1* or *AtMRP2*. Analogs can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a

therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

The invention further includes a transgenic plant comprising an isolated DNA encoding a plant GS-*X* pump polypeptide or a fragment thereof, capable of transporting a glutathionated compound across a biological membrane. The transgenic plant of the invention may comprise a transgene encoding a plant GS-*X* pump polypeptide or a fragment thereof, or it may comprise a transgene encoding a yeast GS-*X* pump polypeptide or a fragment thereof, which yeast transgene is expressed in the plant to yield a biologically active GS-*X* pump protein product. By way of example, there is provided herein in the experimental examples section a transgenic *Arabidopsis* plant comprising a yeast *YCF1* transgene, which when the transgene is expressed in the transgenic plant, confers upon the plant the ability to grow on media containing concentrations of heavy metal (cadmium) or organic xenobiotic (CDNB) that otherwise prevent of nontransgenic plants.

The invention also includes a transgenic plant comprising an isolated DNA comprising the sequence of a plant GS-*X* pump polypeptide or a fragment thereof, which plant GS-*X* pump is capable of transporting a glutathionated compound across a membrane derived from a cell, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

Thus, included in the invention is a transgenic plant comprising an isolated DNA encoding a yeast YCF1 or a fragment thereof, capable of transporting a glutathionated compound across a membrane derived from a cell.

In addition, the invention includes a transgenic plant comprising an isolated DNA comprising the sequence of a yeast *YCF1* gene or a fragment thereof, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

By "transgenic plant" as used herein, is meant a plant, the cells, the seeds and the progeny of which comprise a gene inserted therein, which gene has been manipulated to be inserted into the cells of the plant by recombinant DNA technology. The manipulated gene is designated as a "transgene."

By the term "nontransgenic but otherwise substantially homozygous wild type plant" as used herein, is meant a nontransgenic plant from which the transgenic plant was generated.

"Positioned in an antisense orientation with respect to the direction of transcription of the DNA" as used herein, means that the transcription product of the DNA, the resulting mRNA, does not encode a GS-*X* pump. Rather, the mRNA comprises a sequence which is complementary to an mRNA which encodes a GS-*X* pump.

If vacuolar transport rate limits xenobiotic detoxification and if the amount of GS-*X* pump is rate limiting on the overall rate of vacuolar uptake, transgenic plants with increased *YCF1*, *AtMRP1* or *AtMRP2* expression are expected to be more resistant to the toxic effects of glutathione-conjugable xenobiotics and capable of accumulating higher vacuolar conjugate levels than non-transgenic plants. The former property permits the sustained growth of transgenic plants in the presence of xenobiotic concentrations that would retard the growth of plants exhibiting normal levels of transporter expression. The latter property confers on the plants the ability for hyperaccumulation of glutathionated xenobiotics.

Increased resistance to xenobiotics has application in herbicide technology and plant growth in habitats polluted with organics. Hyperaccumulation has application in the extraction of organic pollutants from contaminated ground soils.

The closest known similar technologies to those described herein (a) involve the isolation of mutants or the engineering of plants in which the target for xenobiotic action is no longer sensitive, (b) involve the generation of mutants with elevated cellular levels of glutathionine (GSH) or with increased glutathione-*S*-transferase activities, or (c) involve the application of chemical agents ("safeners") that

elevate GSH and/or glutathione-*S*-transferase levles or activities. These known technologies differ from the strategy proposed herein in three respects: (i) The utility of mutated target gene products is limited in its application to those xenobiotics that directly interact with the target in question. In contrast, the vacuolar GS-*X* pump is of broad substrate specificity. (ii) Technologies based on elevated cellular GSH levels or increased glutathione-*S*-transferase catalytic efficiencies are limited by the capacity of cells to subsequently metabolize and/or sequester the conjugates generated. The success of these latter technologies eventually depends on delivery of GSH-conjugates into the vacuole and in turn, depends on the activity of the vacuolar GS-*X* pump. (iii) Since the plant vacuole frequently constitutes 40-90% of total intracellular volume and the GS-*X* pump mediates the uptake of xenobiotics into this compartment, the potential for hyperaccumulation on a tissue weight basis is great. Hyperaccumulators may therefore be used for the fixation/sequestration of toxins and their removal from soils. None of the other known technologies have this characteristic.

The generation of transgenic plants comprising sense or antisense DNA having the sequence of a GS-*X* pump or a fragment thereof, may be accomplished by transformation of the plant with a plasmid encoding the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids containing a sense or antisense strand placed under the control of the strong constitutive CaMV 35S promoter or under the control of an inducible promoter (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, Gene 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired gene is known and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Horbor, New York).

Transformation of plants may be accomplished using the Agrobacterium-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual A5*:1).

A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein. Northern blot hybridization analysis or PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the cells of the transgenic plant express GS-*X* polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-*X* pump or fragments thereof. The procedures for performing such analyses are well known in the art and are described, for example, in Sambrook *et al.* (*supra*).

The transgenic plants of the invention are useful for the manipulation of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For example, a transgenic plant encoding an *AtMRP1* or an *AtMRP2* gene or an *AtMRP1*- or *AtMRP2*-related gene, or a yeast *YCF1* or *YCF1*-related gene, is useful for xenobiotic detoxification and heavy metal detoxification when grown on soil containing xenobiotics or heavy metals. Such plants are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

Accordingly, the invention includes a method of removing xenobiotic toxins from soil comprising generating a transgenic plant having a transgene encoding a GS-*X* pump and planting the plant or the seeds of the plant in the soil wherein xenobiotic toxins in the soil are sequestered within the plant during growth of the plant in the soil.

Similarly, the invention includes a method of removing heavy metals from soil comprising generating a transgenic plant having a transgene encoding a GS-*X* pump and planting the plant or the seeds of the plant in the soil wherein heavy metals in the soil are sequestered within the plant during growth of the plant in the soil.

When the levels of xenobiotic toxins or heavy metals in the soil have been sufficiently reduced, the transgenic plant may be removed from the soil and destroyed or discarded in an environmentally safe manner. For example, the harvested plants can be reduced in volume and/or weight by thermal, microbial, physical or chemical means to decrease handling, processing and potential subsequent land filling costs (Cunningham *et al.*, 1996, Plant Physiol. **110**:715-719). In the case of valuable metals, subsequent smelting and recovery of the metal may be cost-effective (Raskin, 1996, *Proc. Natl. Acad. Sci. USA* **93**:3164-3166).

This technique of remediating soil is more efficient, less expensive and easier than most chemical or physical methods. The estimated costs of remediation are as follows: U.S. $10-100 per cubic meter of soil for removal of volatile or water soluble pollutants by *in situ* remediation using plants; U.S. $60-300 per cubic meter of soil for landfill or low temperature thermal treatment remediation of soil contaminated with the same compounds; and, U.S. $200-700 per cubic meter of soil for remediation of soil contaminated with materials requiring special landfilling arrangements or high temperature thermal treatment (Cunningham *et al.*, 1995, *Trends Biotechnol.* **13**:393-397).

Preferably, the transgene in the transgenic plant of the invention is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of AtMRP1, AtMRP2 or YCF1, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2*, *YCF1*.

The types of plants which are suitable for use in this method of the invention include, but are not limited to, high yield crop species for which cultivation practices have already been perfected, or engineered endemic species that thrive in the area to be remediated.

In certain situations, it may be necessary to prevent the removal of substances such as xenobiotic toxins and heavy metals from the soil. In such situations, transgenic plants are generated comprising a transgene comprising a GS-*X* pump sequence which is in the antisense orientation with respect to transcription. Such transgenes therefore serve to inhibit the function of a GS-*X* pump expressed in the plants thereby preventing removal of xenobiotics or heavy metals from the soil.

The production of plants having GS-*X* pump antisense sequences has application in the manipulation of plant/food coloration and in the diminution of organic xenobiotic (*e.g.*, herbicide) or heavy metal accumulation by crop species. For example, ingestion by animals or humans of low organic toxin/low heavy metal crops will likely contribute to an improvement in the overall health of animals and humans.

Accordingly, the invention includes a method of preventing the removal of xenobiotic toxins or heavy metals from soil comprising generating a transgenic plant having a transgene comprising a GS-*X* pump sequence which is in the antisense orientation with respect to transcription and planting the plant or the seeds of the plant in the soil, wherein removal of xenobiotics and heavy metals from the soil is prevented during growth of the plant in the soil.

The antisense sequences which are useful for the generation of transgenic plants having antisense GS-*X* pump sequences are those which will inhibit expression of a resident GS-*X* gene in the plant.

The types of plants which are suitable for use in this method of the invention using antisense sequences include, but are not limited to, plants for which anthocyanins contribute to flower, fruit or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable.

In a similar manner to that described herein, a transgenic plant may be generated which exhibits increased accumulation and/or resistance to isoflavonoid alexins by introducing into the cells of the plant a transgene encoding a GS-*X* pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant, thereby isolating the isoflavonoid alexins from the cytoplasm of the cells of the plant.

Preferably, the transgene is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of AtMRP1, AtMRP2 or YCF1, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2* or *YCF1*.

The invention thus includes a method of generating a pathogen-resistant transgenic plant comprising introducing into the plant a transgene encoding a GS-*X* pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant.

The types of plants suitable for the introduction of the desired transgene include, but are not limited to, plants which are leguminous plants, for example, alfalfa, cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut.

As discussed herein, it has also been discovered in the present invention that the *Bz2* gene which encodes a glutathione-*S*-transferase, glutathionates anthocyanins and possibly other compounds for transport by the GS-*X* pump. The anthocyanin-derivatives so generated are subsequently transported across biological membranes by the vacuolar GS-*X* pump. Vacuolar anthocyanins are responsible for the red and purple hues of many plant organs (petals, leaves, stems, seeds, fruits, etc.). Vacuolar anthocyanins are found in most flowering plants. However, they are not solely responsible for plant coloration. Rather, plant coloration is determined by the relative amounts and combinations in which these various pigments are accumulated. Thus, it is possible to manipulate plant coloration by generating transgenic plants with increased (sense DNA) or decreased (antisense DNA) expression of the GS-*X* pump. Transgenic plants having GS-*X* pump sense sequences are expected to contain more red/purple pigmentation that their nontransgenic but otherwise homozygous counterparts and transgenic plants having GS-X pump antisense sequences are expected to contain less red/purple pigmentation and possibly more brown pigmentation that their nontransgenic but otherwise homozygous counterparts. The generation of such types of transgenic plants may be accomplished following the procedures described herein.

With respect to the aforementioned information regarding anthocyanins, it is important to note that accumulating evidence from studies of the MRP-subclass members from non-plant sources reveals that the group of transporters formerly referred to an GS-*X* pumps because of their affinity toward GS-conjugates, GSSG and cysteinyl leukotrienes, do not transport GS-conjugates exclusively (Ishikawa et al., 1997, Bioscience Reports 17:189-208). Investigation of the human MRP1 protein, cMOAT and ScYCF1 establish that these proteins are capable of transporting a broad range of compounds in addition to GS-conjugates and GSSG Jedlitschky et al., 1996, Cancer Res. 56:988-994; Paulusma et al., 1996, Science 271:1126-1128; Jansen et al., 1987, Hepatol. 7:71-76; Sathirakul et al., 1993, J. Pharmacol. Exp. Therap. 268:65-73). Thus, these proteins transport non-glutathionated compounds.

It has been discovered in the present invention that the plant proteins, AtMRP1 and AtMRP2, differ in their substrate preferences. For example, no only does AtMRP2 exhibit a much higher transport capacity than does AtMRP1, but AtMRP2 has the capacity to transport chlorophyll breakdown products in leaf senescence, which breakdown products are not glutathionated. Thus, according to the present invention, it is possible to manipulate plant coloration by changing the relative levels of expression of various members of this class of transporters in a plant cell. It is possible, using the information provided herein, to affect the rate of breakdown of chlorophyll, for example, by manipulating the expression of AtMRP2 in a plant cell.

In addition to the above, there is provided as part of the invention, *AtMRP1* and *AtMRP2* promoter sequences. By operably coupling the *AtMRP1* or *AtMRP2* promoters to other genes, it may be possible to confer on these other genes expression characteristics similar to those of *AtMRP1* or *AtMRP2*, namely, modulation by xenobiotics, plant pathogens, etc. The data which are presented herein include the promoter sequences of these genes, which promoter sequences are useful in a variety of applications in plants. For example, GS-*X* pump activity which is associated with herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions

(exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins) may be examined as a result of the present invention. The present invention facilitates the identification of plants and cells therein which are capable of GS-*X* pump activity, and further facilitates the exploitation of plant cell GS-*X* pump activity for the purpose of affecting plant function with respect to herbicide metabolism, heavy metal sequestration, plant-pathogen interactions, plant cell pigmentation and plant hormone metabolism.

The invention includes an isolated DNA comprising a plant GS-*X* pump promoter sequence capable of driving expression of a plant GS-*X* pump gene, which gene is capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell.

Preferably, the isolated DNA comprising a plant GS-*X* pump promoter sequence is at least about 40% homologous to at least one of the *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. More preferably, the isolated DNA comprising a plant GS-*X* pump promoter sequence is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at least one of *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. Most preferably, the isolated DNA comprising a plant GS-*X* pump promoter sequence is *Arabidopsis AtMRP1* or *AtMRP2* as shown in Figures 1 and 2, respectively.

Thus, the invention should be construed to include isolated DNA sequences comprising promoter sequences which in their natural form drive expression of genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes. Once armed with the present invention, it is a simple matter to isolate sequences which are related to those shown in Figures 1 and 2. For example, conventional hybridization technology and/or PCR technology may be

employed, primers may be designed using the sequences provided herein, data bases may be searched and the like. Procedures for the isolation of promoter sequences which are related to those described herein are described in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor*, NY) and in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

By the term "promoter sequence" as used herein, is meant a DNA sequence which is required for expression of a gene which is operably linked thereto. In some instances, this sequence may be a core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene in a tissue-specific manner. Thus, a promoter sequence must include an RNA polymerase binding site and may include appropriate transcription factor binding sites as are necessary for activation of transcription and expression of the gene to which the promoter sequence is attached at the 5' end of the gene.

Typically, the promoter sequence of the invention comprises at least about 150 bp in length. More typically, the promoter sequence comprises at least about 300 bp in length. More typically, the promoter sequence comprises at least about 400 bp, even more typically, at least about 500 bp, yet more typically, at least about 600 bp, even more typically, at least about 800 bp, yet more typically, at least about 1000 bp and even more typically, at least about 1200 or more bp in length.

The promoter sequence of the invention may also comprise discrete sequences (elements) which function to regulate the activity of the promoter. Frequently, such elements respond to the presence or absence of environmental factors, thereby controlling gene expression in direct response to factors which are associated with the environmental mileau of the plant. The response of the plant to these factors affects the overall well-being of the plant. Elements which may be present in the promoter sequence of the invention include, but are not limited to, a Myb recognition sequence, a xenobiotic regulatory element, an antioxidant response element, a bZIP recognition sequence, and the like.

Plants from which *AtMRP1*- or *AtMRP2*-related genes and therefore promoter sequences, may be isolated include any plant in which the GS-*X* pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

The invention further includes a vector comprising a plant GS-*X* pump promoter sequence operably fused to a reporter gene and capable of driving expression of the reporter gene. The procedures for the generation of a vector comprising a plant GS-*X* pump promoter sequence are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*) . Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (*e.g.*, pBIN19) (Lagrimini *et al.*, 1990, *Plant Cell* 2:7-18; Bevan, 1984, *Nucl. Acids Res.* 12:8711-8721).

Also included in the invention is a cell comprising a plant GS-*X* pump promoter sequence operably fused to a reporter gene. The procedures for the generation of a cell encoding a plant GS-*X* pump or fragment thereof, are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable cells include, but are not limited to, plant cells, yeast cells, bacterial cells, mammalian cells, and baculovirus-infected insect cells. In addition, plant cells transformed with the promoter/reporter gene construct, for the purpose of assessing the effect of various compounds on promoter activity are also contemplated in the invention. Normal plant cells and those plant cells having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration, which cells comprise the promoter sequence of the invention operably fused to a reporter gene, are all contemplated as part of the invention. When the promoter is fused to a reporter gene, the promoter is said to be operably linked to the reporter gene.

A "reporter gene" as used herein, is one which when expressed in a cell, results in the production of a detectable product in the cell. The level of expression the

product in the cell is proportional to the activity of the promoter sequence which drives expression of the reporter gene.

By describing two nucleic acid sequences as "operably linked" as used herein, is meant that a single-stranded or double-stranded nucleic acid moiety comprises each of the two nucleic acid sequences and that the two sequences are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

Suitable reporter genes include, but are not limited to, β-glucuronidase (GUS) and green fluorescent protein (GFP), although any reporter gene capable of expression and detection in plant cells which are either known or heretofore unknown, may be fused to the plant GS-*X* promoter sequences of the invention.

The invention further includes a transgenic plant comprising an isolated DNA comprising a plant GS-*X* pump promoter sequence as defined herein.

The generation of transgenic plants comprising a plant GS-*X* pump promoter sequence operably fused to a reporter gene, may be accomplished by transformation of the plant with a plasmid comprising the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene* 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired nucleic acid is known and are described, for example, in Ausubel et al. (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the *Agrobacterium*-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual* A5:1).

A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein. Northern blot hybridization analysis or RT-PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the cells of the transgenic plant express GS-*X* polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-*X* pump or fragments thereof. The procedures for performing such analyses are well know in the art and are described, for example, in Sambrook et al. (*supra*).

The transgenic plants of the invention are useful for the examination of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For example, a transgenic plant comprising an *AtMRP1* or an *AtMRP2* promoter sequence fused to a reporter gene is useful for the examination of xenobiotic detoxification and heavy metal detoxification when grown on soil having xenobiotic toxins or heavy metals. Such plants are useful to an understanding of the mechanisms by which GS-*X* pump gene expression is activated and are therefore useful for the eventual generation of plants which are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

The types of plants which are suitable for use include, but are not limited to, high yield crop species for which cultivation practices have already been perfected, or engineered endemic species that thrive in the area to be remediated. In addition plants for which anthocyanins contribute to flower or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable are also suitable for use in the invention. Further useful plants are those in which it is desirable that they are capable of increased accumulation and/or resistance

to isoflavonoid alexins. Plants for which pathogen resistance is desired are also useful in the invention. Such plants include, but are not limited to, plants which are leguminous plants, for example, alfalfa, cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut. In addition, plants for which it is desirable to manipulate plant coloration are also useful in the invention.

The promoter sequences of *Arabidopsis* GS-*X* pump genes *AtMRP1* and *AtMRP2* are shown in Figures 23 and 24, respectively. The following should be noted. bZIP transcription factor recognition elements have the sequences CACGTG or TGACG(T/C). One of these is present in the *AtMRP2* promoter sequence, but none are present in the *AtMRP1* promoter sequence. Myb transcription factor recognition elements having the sequences A(a/D)(a/D)C(G/C) and AGTTAGTTA, wherein a/D = A, G or T with A being preferred, are present in the *AtMRP1* promoter sequence, but are not present in the *AtMRP2* promoter sequence. Xenobiotic regulatory elements (XREs) having the core sequence GCGTG are found in multiple copies in the promoters of cytochrome P450 monooxygenase genes and glutathione *S*-transferase genes (Rushmore et al., 1993, J. Biol. Chem. 268:11475-11478). One XRE is found in the promoter sequence of *AtMRP1*. Antioxidant response elements (AREs) consist typically of two core sequences GTGACA(A/T)(A/T)GC (SEQ ID NO:11) that are binding sites for Activator Protein-1 (AP-1) transcription factor complex (Daniel, 1993, CRC Crit. Rev. Biochem 25:173-207; Friling et al., 1992, Proc. Natl. Acad. Sci. USA 89:668-672). There is only one ARE in the *AtMRP1* promoter sequence shown in Figure 23. It has been proposed that GST genes containing an ARE are induced by electrophiles and conditions that generate oxidative stress (Daniel, *supra*). RNA instability determinants having the sequence ATTTA have been found in several plant GSTs. These sequences, considered to target RNAs for degradation by RNases are usually found in the 3'-UTRs of genes (Takahashi et al., 1992, Proc. Natl. Acad. Sci. USA 89:56-59). Several of these sequences are found in both the *AtMRP1* and *AtMRP2* promoter sequences presented herein. However, it is not clear whether these sequences merely reflect the AT-richness of the sequences.

To assess GS-*X* pump gene expression in a plant cell whether the cell is contained within a plant or whether the cell is separated from the plant, a plasmid may be generated which comprises the β-glucuronidase (GUS) reporter gene fused to a plant GS-*X* promoter sequence. Preferably, the promoter sequence is either *AtMRP1* or *AtMRP2*. The appropriate restriction fragment is subcloned into the GUS expression vector pBI101.3 (Jefferson et al., 1987, EMBO J., 6:3901-3907). After confirming the correct reading frame by sequencing, *Agrobacterium* or any other suitable vector, is transformed with the expression construct and is then used to used to transform the plant, or the cells thereof (Valvekens et al., 1988, Proc. Natl. Acad. Sci. USA 85:5536-5540).

Expression of GUS may be localized histochemically by staining with 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc) (Jefferson et al., *supra*). Sections are obtained from the plant, they are incubated in X-Gluc, cleared by boiling in ethanol and are examined under the microscope. To eliminate or enumerate complications arising from the transfer of GUS reaction product between cells, the distribution of GUS expression is then further examined both immunologically and biochemically. β-glucuronidase protein is assessed using standard dot-blotting and immunolocalization techniques (Harlow et al., 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY) using rabbit anti-β-glucuronidase serum (Clontech). Direct estimates of GUS activity are be made fluorimetrically using 4-methyl-umbelliferyl glucuronide as substrate (Jefferson et al., *supra*) after dissection and extraction of explants.

GUS reporter gene analyses enable examination of plant responses to oxidative stress and pathogens as well as herbicides. In addition, GUS reporter gene analyses enable tests of whether certain pigment-rich cell types also exhibit high levels of AtMRP expression.

The A*tMRP1* and *AtMRP2* promoter sequences are also useful for manipulating the expression of other genes in plants in that, transgenic plants may be generated which contain a desired plant gene operably fused to a GS-*X* pump promoter

sequence. The GS-*X* pump promoter sequence may be an *AtMRP1* or an *AtMRP2* promoter sequence or a *YCF1* promoter sequence positioned in an orientation such that the promoter sequence drives expression of the desired gene. The desired gene may be a plant or a non-plant gene. The generation of such transgenic plants confers upon the plants the ability to respond to the presence of xenobiotics and other compounds which influence the promoter activity

In considering transport substrates for GS-*X* pumps, the status of GSSG as an endogenous GS-conjugate (of GSH with itself) and its involvement in cellular responses to active oxygen species (AOS) should not be overlooked. The sulfhydryl group of GSH confers strong nucleophilicity and the facility for reacting with AOS, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide. GSH is found in the majority of eukaryotes but in prokaryotes (eubacteria) it appears to be restricted to the cyanobacteria and purple bacteria (Fahey and Sundquist 1991, Adv. Enzymol. Relat. Mol. Biol. 64:1-53). Since the cyanobacteria are considered to be the first group of organisms capable of oxygenic photosynthesis and these and the purple bacteria probably gave rise to plant chloroplasts and mitochondria, respectively, it has been proposed that the emergence of the capacity for GSH biosynthesis was associated with the appearance of oxygenic and oxytrophic metabolism (approximately 4×10^9 years ago) to combat the attendant problem of AOS production. Most, if not all, of the factors known to elicit GST induction - pathogen attack, heavy metals, certain organic xenobiotics, wounding and ethylene - promote AOS production (Inze and Montagu 1995, Current Opinion in Biotech. 6:153-158). Intriguing, therefore, is the possibility that GS-*X* pumps arose from the need to detoxify AOS and the products of their action.

The feasibility of such a scheme has yet to be investigated systematically but a number of disparate observations are at least consistent with a close connection between oxidative stress and GS-*X* pump function: (i) All identified MRP-subclass transporters, including AtMRP1 and AtMRP2 recognize GSSG as a substrate. Studies of GS-*X* pumps originated from the discovery of ATP-dependent

GSSG efflux from erythrocytes (Srivastava and Beutler 1969, J. Biol. Chem. 244:9-16). (ii) In *S. cerevisiae*, overexpression of yAP1, a bZIP transcription factor, not only activates the YCF1 and GSH1 genes (Wemmie et al 1994, *supra*; Wu and Moye-Rowley 1994, *supra*), the latter of which encodes γ-glutamylcysteine synthetase, but also a panoply of oxidoreductases (DeRisi et al 1997, Science 278:680-686). Of the 17 genes whose mRNA levels are found to be increased by more than threefold on DNA microarrays by yAP1, more than two-thirds contain canonical upstream yAP1-binding sites (TTACTAA or TGACTAA), five bear homology to aryl-alcohol oxidoreductases and four to the general class of dehydrogenases/oxidoreductases (DeRisi et al 1997, *supra*). In view of the capacity of yAP1 overexpression to confer increased resistance to hydrogen peroxide, o-phenanthroline and heavy metals (Hirata et al 1994, Mol. Gen. Genet. 242:250-257), the fact that an appreciable fraction of the yAP1-regulated target genes identified against the yeast genome project database are oxidoreductases and coregulated with both *YCF1* and *GSH1*, suggests that all of these genes play a protective role during oxidative stress. (iii) Two particularly harmful and early effects of AOS production are membrane lipid peroxidation. and oxidative DNA damage which yield highly toxic 4-hydroxyalkenals (Esterbauer et al 1991, Biochem. J. 208:129-140) and base propanols (Berhane et al 1994, Proc. Natl. Acad. Sci. USA 91:1480-1484), respectively. Although such a,b-unsaturated aldehydes (and their GS-conjugates) have not yet been screened against the GS-*X* pumps from plant sources, they are established substrates for mammalian GSTs (Berhane et al 1994, *supra*) and their glutathionated derivatives are transported at high efficiency by mammalian GS-*X* pumps (Ishikawa 1989, J.Biol. Chem. 264:17343-17348).

There is therefore also included in the invention a method of alleviating oxidative stress in a plant comprising intorducing into the cells of the plant DNA encoding a GS-*X* pump.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of

illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Experimental Examples

The experimental examples described herein provide procedures and results for the isolation and characterization of yeast *YCF1* and *Arabidopsis AtMRP1* and *AtMRP2* genes, gene products and various functions ascribed thereto. Further there is described data which establish that the *Bz2* gene product exerts its effects on plant coloration via the GS-*X* pump.

The data which are now described establish that *YCF1* is a vacuolar glutathione *S*-conjugate pump. The data establish that YCF1 is a membrane protein which is responsible for catalyzing MgATP-dependent, uncoupler-insensitive uptake of glutathione *S*-conjugates into the vacuole of wild type *S. cerevisiae*.

YCF1 encodes a protein responsible for resistance of yeast to the effects of cadmium. However, the mechanism by which resistance to Cd^{2+} is effected was not understood until the present invention. The data presented herein demonstrate that *YCF1* confers Cd^{2+} resistance to yeast by effecting transport of Cd^{2+} out of the cytosol via a *YCF1* encoded vacuolar glutathione *S*-conjugate pump. Further, since *YCF1* confers resistance to Cd^{2+} through the transport of Cd.GS complexes or derivatives thereof, it is likely also capable of transporting other metal.GS-complexes. Examples of these other complexes include, but are not limited to, mercury (Hg), zinc (Zn), platinum (Pt) and arsenic (Ar). Both Hg^{2+} and Zn^{2+} form complexes with GSH which are analogous to those formed by Cd^{2+} (Li *et al.*, 1954, *J. Am. Chem. Soc.* 76:225-229; Kapoor *et al.*, 1965, *Biochem. Biophys. Acta* 100:376-383; Perrin *et al.*, 1971, *Biochem. Biophys. Acta* 230:96-104). In addition, MRP1 eliminates the Pt^{2+} glutathionine complex *bis*(glutathionato)platinum from cancer cells (Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). Further, the MRP1 gene is overexpressed in cisplatin-resistant human leukemia HL-60 cells, which overexpression is associated

with increased resistance to arsenite (Ishikawa *et al.*, 1996, *J. Biol. Chem.* 271:14981-14988). Both Hg^{2+} and Ar^{2+} are common environmental contaminants and Zn^{2+} is an essential micronutrient.

According to the results of the present study, vacuolar membrane vesicles from wild type *S. cerevisiae* catalyze high rates of MgATP-dependent, uncoupler-insensitive *S*-conjugate transport, and the kinetics of the transporter involved are similar to those of the mammalian and plant vacuolar GS-*X* pumps. In addition, vacuole-deficient mutants of *S. cerevisiae* exhibit markedly increased sensitivity to cadmium, leading to the belief that one requirement for efficient elimination or detoxification of this metal is maintenance of a sizable vacuolar compartment.

It is known that *S. cerevisiae* yAP-1 transcription factor transcriptionally activates both the *YCF1* gene and the *GSH1* gene (Wemmie *et al.*, 1994, *J. Biol. Chem.* 269:32592-32597; Wu *et al.*, 1994, *Mol. Cell. Biol.* 14:5832-5839). Since *GSH1* encodes γ-glutamylcysteine synthetase, an enzyme critical for GSH synthesis, expression of the *YCF1* gene and fabrication of one of the precursors for transport by the GS-*X* pump are coordinately regulated.

In the first set of experiments described below, transport of the model compounds DNP-GS and bimane-GS by isolated membrane vesicles and intact cells was examined.

Yeast Strains and Plasmids

Two strains of *S. cerevisiae* were used in these studies: DTY165 (*MATα ura3-52 his6 leu2-3,-112 his3-Δ200 trp1-901 lys2-801 suc2-Δ*) and the isogenic *ycf1Δ* mutant strain, DTY167 (*MATα ura 3-52 his6 leu2-3,-112 his3-Δ200 trp 1-901 lys2-801 suc2-Δ, ycf1::hisG*). The strains were routinely grown in rich (YPD) medium, or, when transformed with plasmid containing functional *YCF1* gene, in synthetic complete medium (Sherman *et al.*, 1983, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, New York) or AHC medium (Kim *et al.*, 1994, *supra*) lacking the appropriate amino acids. *Escherichia coli* strains XL1-blue (Stratagene)

and DH11S were employed for the construction and maintenance of plasmid stocks (Ausubel *et al.*, 1987, *Current Protocols in Molecular Biology*, Wiley, New York).

Plasmid pYCF1-HA, encoding epitope-tagged *YCF1*, was constructed in several steps. A 1.4-kb *Sal*I-*Hind*III fragment, encompassing the carboxyl-terminal segment of the open reading frame of *YCF1*, from pIBIYCF1 (Szczypka *et al.*, 1994, *supra*), was subcloned into pBluescript KS$^-$. Single-stranded DNA was prepared and used as template to insert DNA sequence encoding the human influenza hemagglutinin 12CA5 epitope immediately before the termination codon of the *YCF1* gene by oligonucleotide-directed mutagenesis. The sequence of the primer for this reaction, with the coding sequence for the 12CA5 epitope underlined, was 5'-GTTTCACAGTTTAAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTG ACC-3' (SEQ ID NO:12). After confirming the boundaries and fidelity of the HA-tag coding region by DNA sequencing, the 1.4-kb *Sal*I-*Hind*III DNA fragment was exchanged with the corresponding wild type segment of pJAW50 (Wemmie *et al.*, 1994, *supra*) to generate pYCF1-HA.

Isolation of Vacuolar Membrane Vesicles

For the routine preparation of vacuolar membrane vesicles, 15 ml of stationary phase cultures of DTY165 or DTY167 were diluted into 1-liter volumes of fresh YPD medium, grown for 24 hours at 30°C to an $OD_{600\ nm}$ of approximately 0.8 and collected by centrifugation. After washing with distilled water, the cells were converted to spheroplasts with Zymolyase 20T (ICN) (Kim *et al.*, 1994, *supra*) and intact vacuoles were isolated by flotation centrifugation of spheroplast lysates on Ficoll 400 step gradients as described by Roberts *et al.* (1991, *Methods. Enzymol.* **194**:644-661). Both the spheroplast lysis buffer and Ficoll gradients contained 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF to minimize proteolysis. The resulting vacuole fraction was vesiculated in 5 mM $MgCl_2$, 25 mM KC1, 10 mM Tris-Mes (pH 6.9) containing 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF, pelleted by centrifugation at 37,000 x g for 25 min, and resuspended

in suspension medium (1.1 M glycerol, 2 mM dithiothreitol, 1 mM Tris-EGTA, 2 mg/ml bovine serum albumin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, 5 mM Tris-Mes, pH 7.6) (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635).

In experiments involving cadmium transport, dithiothreitol and EGTA were removed from the suspension medium to prevent the attenuation of YCF1-dependent Cd^{2+} transport otherwise exerted by these compounds. Vesiculated vacuolar membranes were subjected to three cycles of 50-fold dilution into simplified suspension medium (1.1 M glycerol, 5 mM Tris-Mes, pH 8.0), centrifugation at 100,000 x g for 35 minutes and resuspension in the same medium before use.

For the experiment shown in Figure 4, 1 ml of partially purified vacuolar membrane vesicles (1.1-1.2 mg of protein), prepared by Ficoll flotation, were subjected to further fractionation by centrifugation through a 30-ml linear 10-40% (w/v) sucrose density gradient at 100,000 x g for 2 hours. Successive fractions were collected from the top of the centrifuge tube and, after determining sucrose concentration refractometrically, the fractions were diluted with suspension medium. The diluted fractions were sedimented at 100,000 x g and resuspended in 100-μl aliquots of suspension medium for assay. For the immunoblots shown in Figure 5 and the marker enzyme analyses shown in Table 4, crude microsomes were prepared by homogenization of spheroplasts in suspension medium and the sedimentation of total membranes at 100,000 x g for 35 minutes.

Microsomes and purified vacuolar membranes that were to be employed for SDS-polyacrylamide gel electrophoresis and immunoblotting were washed free of bovine serum albumin by three rounds of suspension in suspension medium minus bovine serum albumin and centrifugation at 100,000 x g for 35 minutes. The final membrane preparations were either used immediately or frozen in liquid nitrogen and stored at -85°C.

Measurement of Marker Enzyme Activities

α-Mannosidase was determined according to Opheim (1978, *Biochem. Biophys. Acta* **524**:121-125) using *p*-nitrophenyl-α-D-mannopyranoside as substrate. NADPH-cytochrome *c* reductase was estimated as FMN-promoted reduction of NADPH (Kubota *et al.*, 1977, *J. Biol. Chem.* **81**:197-201). GDPase was measured as the rate of liberation of P_i from GDP (Yanagisawa *et al.*, 1990, *J. Biol. Chem.* **265**:19351-19355) in reaction buffer containing 0.05% (w/v) Triton X-100. V-ATPase, F-ATPase, and P-ATPase were assayed as bafilomycin A_1 (1 μM), azide (1 mM), and vanadate (100 μM) inhibited ATPase activity, respectively, at pH 8.0 (V-ATPase, F-ATPase) or pH 6.5 (P-ATPase) (Rea and Turner, 1990, *Methods Plant Biochem.* **3**:385-405).

Measurement of DNP-GS Uptake

Unless otherwise indicated, [^{3}H]DNP-GS uptake was measured at 25°C in 200 μl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KC1, 1 mg/ml bovine serum albumin, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0), and 66.2 μM [^{3}H]DNP-GS (8.7 mCi/mmol) (Li *et al.*, 1995, *supra*). Gramicidin D was included in the uptake medium to abolish the H^+ electrochemical potential difference ($\Delta\mu_H+$) that would otherwise be established by the V-ATPase in medium containing MgATP. Uptake was initiated by the addition of vacuolar membrane vesicles (10-15 μg of membrane protein), brief mixing of the samples on a vortex mixer and uptake was then allowed to proceed for 1-60 minutes. Uptake was terminated by the addition of 1 ml of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter, 0.45 μm). The filters were rinsed twice with 1 ml of ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in BCS mixture (Amersham Corp.). Nonenergized [^{3}H]DNP-GS uptake and extravesicular solution trapped on the filters were enumerated by the same procedure except that ATP and Mg^{2+} were omitted from the uptake medium.

Fluorescence Microscopy

Cells were grown in YPD medium for 24 hours at 30°C to an $OD_{600\ nm}$ of approximately 1.4, and 100 µl aliquots of the suspensions were transferred to 15 ml volumes of fresh YPD medium containing 100 µM *syn*-($ClCH_2$,CH_3)-1,5-diazabicyclo-[3.3.0]-octa-3,6-dione-2,8-dione (monochlorobimane) (Kosower *et al.*, 1980, *J. Am. Chem. Soc.* 102:4983-4993). After incubation for 6 hours, the cells were pelleted by centrifugation, washed twice with YPD medium lacking monochlorobimane, and viewed without fixation under an Olympus BH-2 fluorescence microscope equipped with a BP-490 UV excitation filter, AFC-0515 barrier filter, and Nomarski optics attachment.

Electrophoresis and Immunoblotting

Membrane samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 7-12% (w/v) concave exponential gradient gels after delipidation with acetone:ethanol (Parry *et al.*, 1989, *J. Biol. Chem.* 264:20025-20032). The separated polypeptides were electrotransferred to 0.45 µm nitrocellulose filters at 60 V for 4 hours at 4°C in a Mini Trans-Blot transfer cell (Bio-Rad) and reversibly stained with Ponceau-S (Rea *et al.*, 1992, *Plant Physiol.* 100:723-732). The filters were blocked and incubated overnight with mouse anti-HA monoclonal antibody (20 µg/ml) (Boehringer-Mannheim). Immunoreactive bands were visualized by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (1/1000 dilution) (Boehringer-Mannheim) and incubation in buffer containing H_2O_2 (0.03% w/v), diaminobenzidine (0.6 mg/ml) and $NiCl_2$ (0.03% w/v) (Rea *et al.*, 1992, *supra*).

Purification of Cadmium-Glutathione Complexes

Singly radiolabeled $^{109}Cd.GS_n$ and doubly radio-labeled $^{109}Cd[^3H].GS_n$ complexes were prepared by sequential gel-filtration and anion-exchange chromatography of the reaction products generated by incubating 20 mM $^{109}CdSO_4$ (78.4 mCi/mmol) with 40 mM GSH or 40 mM [^{3}H]GSH (240 mCi/mmol) in 15 ml 10 mM phosphate buffer (pH 8.0) containing 150 mM KNO_3 at 45°C for 24 hours. For gel-filtration, 2 ml aliquots of the reaction mixture were applied to a column (40 x 1.5 cm ID) packed with water-equilibrated Sephadex G-15, eluted with

deionized water and ^{109}Cd and/or ^{3}H in the fractions was measured by liquid scintillation counting. The fractions encompassed by each of the two $^{109}Cd.GS_n$ peaks identified were pooled, lyophilized and redissolved in 4 ml of loading buffer (5 mM Tris-Mes, pH 8.0). For anion-exchange chromatography, 0.5 ml aliquots of the resuspended lyophilizates from gel-filtration chromatography were applied to a Mono-Q HR5/5 column (Pharmacia) equilibrated with the same buffer. Elution was with a linear gradient of NaCl (0.5 ml/minute; 0-500 mM) dissolved in loading buffer. The individual fractions corresponding to the major peaks of ^{109}Cd obtained from the Mono-Q column (one each for the peaks resolved by gel-filtration chromatography) were pooled, lyophilized and resuspended in 4 ml deionized water after liquid scintillation counting. Buffer salts were removed before transport measurements or mass spectrometry by passing the samples down a column (120 x 1.0 cm ID) packed with water-equilibrated Sephadex G-15.

Measurement of $^{109}Cd^{2+}$ Uptake

MgATP-energized, uncoupler-insensitive $^{109}Cd^{2+}$ uptake by vacuolar membrane vesicles was measured at 25°C in 200 µl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 µM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of $^{109}CdSO_4$, GSH or ^{109}Cd- and/or ^{3}H-labeled purified $Cd.GS_n$ complexes as described herein except that the wash media contained 100 µM $CdSO_4$ in addition to sorbitol (400 mM) and Tris-Mes (3 mM, pH 8.0).

Pretreatment of DTY165 Cells with Cd^{2+} or 1-Chloro-2,4-dinitrobenzene

For studies on the inducibility of *YCF1* expression and YCF1-dependent transport, DTY165 cells were grown in YPD medium (Sherman *et al.*, 1983, *supra*) for 24 hours at 30°C to an $OD_{600\ nm}$ of 1.0-1.2, pelleted by centrifugation and resuspended in fresh YPD medium containing $CdSO_4$ (200 µM) or 1-chloro-2,4-dinitrobenzene (CDNB). After washing in distilled water, total RNA was extracted and vacuolar membrane vesicles were prepared from the pretreated cells.

Control RNA and membrane samples were prepared from DTY165 cells treated in an identical manner except that $CdSO_4$ and CDNB were omitted from the second incubation cycle.

RNase Protection Assays

Cd^{2+} and CDNB-elicited increases in *YCF1* mRNA levels were assayed by RNase protection using 18S rRNA as an internal control. *YCF1*-specific probe was generated by PCR amplification of the full-length *YCF1::HA* gene, encoding human influenza hemagglutinin 12CA5 (HA) epitope-tagged *YCF1*, using plasmid pYCF1-HA as template. The forward *YCF1*-specific primer and backward primer containing the HA-tag coding sequence had the sequences 5'-AAACTGCAGATGGCTGGTAATCTTGTTTC-3' (SEQ ID NO:13) and 5'-GCCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTGA-3' (SEQ ID NO:14), respectively. An 18S rRNA-specific probe was synthesized by PCR of *S. cerevisiae* genomic DNA using sense and antisense primers having the sequences 5'-AGATTAAGCCATGCATGTCT-3' (SEQ ID NO:15) and 5'-TGCTGGTACCAGACTTGCCCTCC-3' (SEQ ID NO:16), respectively. Both PCR products were individually subcloned into pCR™II vector (Invitrogen) to generate plasmids pCR-*YCF1* and pCR-Y18S. After linearization of pCR-*YCF1* and pCR-Y18S with *Afl*II and *Nco*I, a 320-nucleotide *YCF1*-specific RNA probe and 220-nucleotide 18S rRNA-specific probe were synthesized using T7 RNA polymerase and SP6 RNA polymerase, respectively. Aliquots of total RNA, prepared as described (Kohrer *et al.*, 1991, *Methods in Enzymol.* 194:390-398)), from control, $CdSO_4$- or CDNB-pretreated DTY165 cells were hybridized with a mixture of ^{32}P-labeled *YCF1* antisense probe (1×10^6 cpm) and 18S rRNA antisense probe (5×10^2 cpm) and RNase protection (Teeter *et al.* , 1990, *Mol. Cell. Biol.* **10**:5728-5735) was assayed using an RPAII kit (Ambion).

Matrix-Assisted Laser Desorption Mass Spectrometry (MALD-MS)

The $^{109}Cd.GS_n$ complexes purified by gel-filtration and anion-exchange chromatography were adjusted to a final concentration of 2-5 mM (as Cd)

with deionized water, mixed with an equal volume of sinapinic acid (10 mg/ml) dissolved in acetonitrile/$H_2$0/trifluoroacetic acid (70:30:0.1 % (v/v)) and applied to the ion source of a PerSeptive Biosystems Voyager RP Biospectrometry Workstation. The instrument, which was equipped with a 1.3 m flight tube and variable two-stage ion source set at 30 kV, was operated in linear mode. Mass/charge (m/z) ratio was measured by time-of-flight after calibration with external standards.

Protein Estimations

Protein was estimated by a modification of the method of Peterson (1977, *Anal. Biochem.* 83:346-356).

Chemicals

S-(2,4-dinitrophenyl)glutathione (DNP-GS) was synthesized from 1-chloro-2,4-dinitrobenzene (CDNB) and GSH by the procedure of Kunst *et al.* (1983, *Biochem. Biophys. Acta* 983:123-125) and (Li *et al.*, 1995, *supra*). [^{3}H]DNP-GS (specific activity, 8.7 mCi/mmol) and bimane-GS were synthesized enzymatically and purified by a modification of the procedure of Kunst *et al.* (1983, *supra*) according to Li *et al.* (1995, *supra*). Metolachlor-GS was synthesized by general base catalysis and purified by reverse-phase high performance liquid chromatography (Li *et al.*, 1995, *supra*).

GSH and CDNB were purchased from Fluka; AMP-PNP, aprotinin, ATP, creatine kinase (type I from rabbit muscle, 150-250 units/mg of protein), creatine phosphate, FCCP, oxidized glutathione (GSSG), S-methylglutathione, cysteinylglycine, cysteine, glutamate and gramicidin D, leupeptin, PMSF, verapamil, and vinblastine were from Sigma; monochlorobimane was from Molecular Probes; cellulose nitrate membranes (0.45-μm pore size, HA filters) were from Millipore; [^{3}H]glutathione[(glycine-2-^{3}H]-L-Glu-Cys-Gly; 44 Ci/mmol) was from DuPont NEN; and $^{109}CdSO_4$ (78.44 Ci/mmol) was from Amersham Corp. Metolachlor was a gift from CIBA-Geigy, Greensboro, NC. All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

Sensitivity to CDNB

If the *YCF1* gene product were to participate in the detoxification of *S*-conjugable xenobiotics, mutants deleted for this gene would be expected to be more sensitive to the toxic effects of these compounds than wild type cells. This is what was found (Figure 1).

The isogenic wild type strain DTY165 and the *ycf1*Δ mutant strain, DTY167, were indistinguishable during growth in YPD medium lacking CDNB; both strains grew at the same rate after a brief lag. However, the addition of CDNB to the culture medium caused a greater retardation of the growth of DTY167 cells (Figure 1B) than DTY165 cells (Figure 1A). Inhibitory concentrations of CDNB resulted in a slower, more linear, growth rate for at least 24 hours for both strains, but DTY167 underwent growth retardation at lower concentrations than did DTY165. The optical densities of the DTY167 cultures were diminished by 65, 82, 85, and 91% by 40, 50, 60, and 70 μM CDNB, respectively, after 24 hours of incubation (Figure 1B), whereas the corresponding diminutions for the DTY165 cultures were 14, 31, 59, and 92% (Figure 1A). The increase in sensitivity to CDNB conferred by deletion of the YCF1 gene was similar to that seen with cadmium.

Impaired Vacuolar DNP-GS Transport

Vacuolar membrane vesicles purified from DTY165 cells exhibited high rates of MgATP-dependent [^{3}H]DNP-GS uptake (Figure 2). Providing that creatine phosphate and creatine kinase were included in the uptake media to ensure ATP regeneration, addition of 3 mM MgATP increased the initial rate of DNP-GS uptake by 122-fold to a value of 12.2 nmol/mg/minute. The same membrane fraction from DTY167 cells, although capable of similar rates of MgATP-independent DNP-GS uptake, was only 17-fold stimulated by MgATP and capable of an initial rate of uptake of only 1.7 nmol/mg/minute (Figure 2).

Selective Impairment of Uncoupler-Insensitive Transport

Direct comparisons between vacuolar membrane vesicles from DTY165 and DTY167 cells demonstrated that deletion of the *YCF1* gene selectively abolished MgATP-energized, $\Delta\mu_H$+-independent DNP-GS transport.

Agents that dissipate both the pH (ΔpH) and electrical (Δψ) components of the $\Delta\mu_H+$ established by the V-ATPase (FCCP, gramicidin D) or directly inhibit the V-ATPase, itself (bafilomycin A_1), decreased MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles from DTY165 cells from 77.7 ± 1.0 nmol/mg/10 minutes to between 43.2 ± 1.0 and 47.4 ± 1.7 nmol/mg/10 minutes (Table 1). Ammonium chloride, which abolishes ΔpH while leaving Δψ unaffected, on the other hand, did not inhibit DNP-GS uptake (Table 1). On the basis of these characteristics, the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone, and the resistance of 50-60% of total uptake to inhibition by any one of these compounds (Table 1), DNP-GS uptake by vacuolar membranes from wild type cells is concluded to proceed via two parallel mechanisms: a V-ATPase inhibitor- and uncoupler-insensitive pathway that is directly energized by MgATP, and a $\Delta\mu_H+$-dependent, V-ATPase inhibitor-sensitive and uncoupler-sensitive pathway that is primarily driven by the inside-positive Δψ established by the V-ATPase.

Of these two pathways, the Δψ-dependent pathway predominated in membranes from DTY167 cells (Table 1). FCCP, gramicidin D, and bafilomycin A_1 diminished net DNP-GS uptake by DTY167 vacuolar membranes from 15.4 ± 0.4 nmol/mg/10 minutes to between 4.3 ± 0.3 and 6.4 ± 0.3 nmol/mg/10 minutes. Moreover, although the effects of FCCP or gramicidin D and V-ATPase inhibitors in combination were slightly greater than those seen when these agents were added individually, the transport remaining was only about 10% of that seen with wild type membranes and only 2–4-fold stimulated by MgATP. In conjunction with the negligible inhibitions seen with NH_4Cl, alone, indicating that Δψ, not ΔpH, is the principal driving force for the transport activity remaining in their vacuolar membranes, DTY167 cells are inferred to be preferentially impaired in MgATP-energized, $\Delta\mu_H+$-independent DNP-GS transport.

The nonhydrolyzable ATP analog, AMP-PNP, did not promote DNP-GS uptake by vacuolar membrane vesicles from either DTY165 or DTY167 cells

(Table 1), indicating a requirement for hydrolysis of the γ-phosphate of ATP regardless of whether uptake was via the YCF1- or Δψ-dependent pathway.

Table 1. Effects of MgATP, MgAMP-PNP, protonophores, ionophores and V-ATPase inhibitors on [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.

Uptake was measured for 10 minutes in standard uptake medium described herein containing 66.2 μM [^{3}H]DNP-GS plus the compounds indicated. MgATP (3 mM) was present throughout unless otherwise indicated. MgAMP-PNP, bafilomycin A_1, FCCP, gramicidin D, and NH_4Cl were added at concentrations of 3 mM, 0.5 μM, 5 μM, 5 μM and 1 mM, respectively. Values outside parentheses are means ± SE (n = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITIONS	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	77.7 ± 1.0 (100)	15.4 ± 0.4 (100)
-MgATP	2.2 ± 0.4 (2.8)	1.5 ± 0.6 (9.7)
MgAMP-PNP(-MgATP)	2.5 ± 0.5 (3.2)	1.4 ± 0.3 (9.1)
FCCP	47.4 ± 1.7 (61.0)	6.4 ± 0.3 (41.8)
Gramicidin D	45.8 ± 1.4 (58.9)	5.8 ± 0.1 (37.7)
NH_4Cl	69.1 ± 2.9 (88.9)	14.9 ± 0.7 (96.8)
NH_4Cl + gramicidin D	42.6 ± 1.8 (54.8)	4.1 ± 0.2 (26.6)
Bafilomycin A_1	43.2 ± 1.0 (55.6)	4.3 ± 0.3 (27.9)
Bafilomycin A_1 + gramicidin D	39.2 ± 2.6 (50.5)	3.8 ± 0.1 (24.7)

Abolition of High Affinity, Uncoupler-insensitive Uptake

Examination of the concentration dependence of [^{3}H]DNP-GS uptake revealed a near total abolition of high affinity, MgATP-dependent, uncoupler-insensitive transport by vacuolar membrane vesicles from the *ycf1*Δ mutant strain (Figure 3). When measured in the presence of uncoupler (gramicidin D), the rate of DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells increased as a simple hyperbolic function of MgATP (Figure 3A) and DNP-GS concentration (Figure 3B) to yield K_m values OF 86.5 ± 29.5 μM (MgATP) and 14.1 ± 7.4 μM

(DNP-GS) and a V_{max} of 51.0 ± 6.3 nmol/mg/10 minutes (DNP-GS). By contrast, uncoupler-insensitive uptake by the corresponding membrane fraction from DTY167 cells was more than 15-fold slower over the entire concentration range, showed no evidence of saturation and increased as a linear function of both DNP-GS and MgATP concentration (Figure 3).

Selective Inhibitors of YCF1-mediated Transport

MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells was sensitive to inhibition by vanadate, vinblastine, verapamil, GSSG and glutathione *S*-conjugates other than DNP-GS (Tables 2 and 3). One hundred μM concentrations of metolachlor-GS, azidophenacyl-GS and bimane-GS and 1 mM GSSG inhibited uptake by about 50% (Table 2), while vanadate, vinblastine, and verapamil exerted 50% inhibitions at concentrations of 179, 89 and 203 μM, respectively (Table 3). None of these agents significantly inhibited residual MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles from DTY167 cells (Tables 2 and 3).

TABLE 2. Effects of GSH, GSSG, and glutathione *S*-conjugates other than DNP-GS on MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured as described for Table 1 except that 5 μM gramicidin D was included in all of the uptake media. Values outside parentheses are means ± SE (*n* = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.

COMPOUND	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	47.9 ± 2.5 (100)	6.5 ± 0.8 (100)
GSH (1 mM)	50.6 ± 2.3 (105.6)	4.6 ± 1.1 (70.8)
GSSG (1 mM)	26.0 ± 0.9 (54.3)	4.4 ± 0.4 (67.7)
Metolachlor-GS (100 μM)	27.6 ± 0.9 (57.7)	4.8 ± 0.7 (73.8)
Azidophenacyl-GS (100 μM)	16.0 ± 1.4 (33.5)	5.2 ± 0.3 (80.0)
Bimane-GS (100 μM)	25.2 ± 1.1 (52.6)	4.5 ± 0.4 (69.2)

TABLE 3. Sensitivity of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells to inhibition by vanadate, vinblastine, and verapamil. Uptake was measured as described in Table 1 except that 5 μM gramicidin D was included in all of the uptake media. The concentrations of the compounds causing 50% inhibition of uptake (I_{50} values) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential (Marquardt, 1963, *supra*).

Addition	I_{50}	
	DTY165	DTY167
	μM	
Vanadate	179.1	Insensitive
Vinblastine	88.8	> 500
Verapamil	202.6	Insensitive

Vacuolar Membrane Localization

The capacity for MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake strictly copurified with the vacuolar membrane fraction (Table 4). By

comparison with crude microsomes (total membranes) prepared from whole spheroplast homogenates of DTY165 cells, vacuolar membrane vesicles derived from vacuoles purified by the Ficoll flotation technique were coordinately enriched for DNP-GS uptake and for both of the vacuolar membrane markers assayed, α-mannosidase and bafilomycin A_1-sensitive ATPase (V-ATPase) activity. The respective enrichments of MgATP-dependent, uncoupler-insensitive DNP-GS uptake, α-mannosidase and bafilomycin A_1-sensitive ATPase activity were 28-, 53- and 22-fold. By contrast, the vacuolar membrane fraction was 4.5-, 6.3-, 11.1- and 4.3-fold depleted of NADPH cytochrome *c* reductase (endoplasmic reticulum), latent GDPase (Golgi), vanadate-sensitive ATPase (plasma membrane), and azide-sensitive ATPase activity (mitochondrial inner membrane), respectively. Accordingly, when vacuolar membrane vesicles derived from Ficoll-flotated vacuoles were subjected to further fractionation on linear 10–40% (w/v) sucrose density gradients, MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake, α-mannosidase and bafilomycin A_1-sensitive ATPase activity were found to comigrate and exhibit identical density profiles (Figure 4).

TABLE 4. Comparison of rates of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS transport and specific activities of marker enzymes in crude microsomes and vacuolar membrane vesicles prepared from DTY165 cells. Microsomes and vacuolar membrane vesicles were prepared from spheroplasts and the marker enzymes were assayed as described herein. Values shown are means ± SE (n = 3).

PREPARATION	ACTIVITY		
	DNP-GS UPTAKE	α-mannosidase	NADPH-cyt c reductase
	nmol/mg/10 min	*nmol/mg/min*	*nmol/mg/min*
Microsomes	2.5 ± 0.3	6.3 ± 0.3	88.0 ± 1.3
Vacuolar membrane	69.9 ± 1.0	329.3 ± 3.2	19.3 ± 0.6
Enrichment (-fold)	27.96	52.27	0.22

PREPARATION	ACTIVITY			
	V-ATPase	GDPase	P-ATPase	F-ATPase
	μmol/mg/h		*μmol/mg/h*	
Microsomes	11.7 ± 6.3	35.0 ± 1.1	37.1 ± 4.6	155.6 ± 3.0
Vacuolar membrane	253.1 ± 15.8	5.5 ± 0.1	3.2 ± 1.6	35.1 ± 8.4
Enrichment (-fold)	21.63	0.16	0.09	0.23

Plasmid-encoded YCF1 Mediates Vacuolar DNP-GS Transport and CDNB Resistance

Immunoblots of vacuolar membranes from pYCF1-HA-transformed DTY165 or DTY167 cells, probed with mouse anti-HA monoclonal antibody, demonstrated incorporation of YCF1-HA polypeptide into the vacuolar membrane fraction (Figure 5B). Immunoreaction with the 12CA5 epitope was not detectable in lanes loaded with membranes from pRS424-transformed cells but the same quantities of membranes prepared from pYCF1-HA-transformed cells yielded a single intensely immunoreactive band with an electrophoretic mobility (M_r = 156,200) commensurate

with a computed mass of 172 kDa for the fusion protein encoded by YCF1-HA (Figure 5B).

Direct participation of the plasmid-borne YCF1-HA gene product in DNP-GS transport and CDNB detoxification was verified by the finding that vacuolar membrane vesicles purified from pYCF1-HA-transformed DTY167 cells exhibited a 6-fold enhancement of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake (Figure 5A) which was accompanied by a decrease in the susceptibility of such transformants to growth retardation by exogenous CDNB (Figure 6). Whereas pYCF1-HA-transformed DTY167 cells exhibited a similar resistance to growth retardation by CDNB as untransformed DTY165 cells (compare Figure 6B with Figure 1A), the same mutant strain showed neither increased vacuolar DNP-GS transport *in vitro* nor decreased susceptibility to CDNB *in vivo* after transformation with parental plasmid pRS424, lacking the YCF1-HA insert (Figure 6B).

Vacuolar Accumulation of Bimane-GS *In Vivo*

Monochlorobimane, a membrane-permeant, nonfluorescent compound, is specifically conjugated with GSH by cytosolic glutathione *S*-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant *S*-conjugate, bimane-GS (Shrieve *et al.*, 1988, *J. Biol. Chem.* **263**:14107-12114; Oude Elferink *et al.*, 1993, *Hepatology* 17:343-444; Ishikawa *et al.*, 1994, *J. Biol. Chem.* **269**:29085-29093). The GS-*X* pumps of both animal and plant cells exhibit activity toward a broad range of *S*-conjugates, including bimane-GS (Ishikawa *et al.*, 1994, *supra*; Martinoia *et al.*, 1993, *supra*), and DNP-GS uptake by the yeast enzyme is shown herein to be reversibly inhibited by this compound (Table 2). These data suggest competition between bimane-GS and DNP-GS for a common uptake mechanism. Exogenous monochlorobimane therefore satisfies the minimum requirements of a sensitive probe for monitoring the intracellular transport and localization of its *S*-conjugate.

Fluorescence microscopy of DTY165 and DTY167 cells after incubation in growth medium containing monochlorobimane provides direct evidence that YCF1 contributes to the vacuolar accumulation of its glutathione *S*-conjugate by

intact cells (Figure 7). DTY165 cells exhibited an intense punctate fluorescence, corresponding to the vacuole as determined by Nomarski microscopy, after 6 hours of incubation with monochlorobimane (Figures 7A and 7C). The fluorescence associated with vacuolar bimane-GS was by comparison severely attenuated in most, and completely absent from many, DTY167 cells (Figures 7B and 7D).

*ycf1*Δ Mutants are Defective in GSH-Dependent Cd^{2+} Transport

Physiological (1 mM) concentrations of GSH (Kang, 1992, *Drug Metabolism and Disposition* 20:714-718) promoted Cd^{2+} uptake by vacuolar membrane vesicles purified from the wild type strain DTY165 but not the *ycf1*Δ mutant strain DTY167 (Figure 8). Addition of Cd^{2+} (80 μM) to GSH-containing media elicited MgATP-dependent, uncoupler-insensitive $^{109}Cd^{2+}$ uptake rates of 4.5 and 0.8 nmol/mg/minute by DTY165 and DTY167 membranes, respectively (Figures 8A and 8B). Uptake by DTY165 membranes was diminished more than 9-fold by the omission of GSH (Figure 8A) whereas uptake by DTY167 membranes was slightly stimulated (Figure 8B).

GSH maximally stimulated uptake within minutes ($t_{1/2} < 5$ minutes) of the addition of Cd^{2+} to the uptake medium and uptake was sigmoidally dependent on Cd^{2+} concentration, achieving half-maximal velocity at 120 μM (Figure 8C).

Specific Requirement for GSH

The stimulatory action of GSH was abolished by the omission of MgATP from the assay medium (Figure 8 and Table 5) and 1 mM concentrations of GSSG, *S*-methylglutathione, cysteinylglycine, cysteine or glutamate did not promote MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by vacuolar membrane vesicles from either strain (Table 5).

TABLE 5. Effects of different GSH-related compounds on uncoupler-insensitive ^{109}Cd uptake by vacuolar membrane vesicles purified from DTY165 or DTY167 cells. GSH, oxidized glutathione (GSSG), *S*-methylglutathione (GS-CH_3), cysteinylglycine, cysteine and glutamate were added at concentrations of 1 mM. MgATP, $^{109}CdSO_4$ and gramicidin-D were added at concentrations of 3 mM, 80 μM and 5 μM, respectively. Values shown are means ± SE (n = 3-6).

COMPOUND	^{109}Cd UPTAKE (*nmol/mg/10 minutes*)			
	DTY165		DTY167	
	-MgATP	+MgATP	-MgATP	+MgATP
Cd^{2+}	5.8 ± 2.4	5.6 ± 1.5	4.3 ± 1.3	4.6 ± 2.1
Cd^{2+} + GSH	4.2 ± 1.2	37.4 ± 4.5	3.3 ± 1.1	8.3 ± 2.7
Cd^{2+} + GSSG	-	5.1 ± 3.2	-	3.8 ± 2.3
Cd^{2+} + GS-CH_3	-	4.5 ± 1.9	-	3.7 ± 3.1
Cd^{2+} + Cys-Gly	-	5.6 ± 3.2	-	6.9 ± 1.4
Cd^{2+} + Cys	-	7.0 ± 1.2	-	3.9 ± 1.0
Cd^{2+} + Glu	-	5.7 ± 1.1	-	5.2 ± 1.3

Purification of Transport-Active Complex

To determine the mode of action of GSH and the form in which Cd^{2+} is transported, reaction mixtures initially containing Cd^{2+} and GSH were fractionated and *YCF1*-dependent uptake was assayed.

Incubation of $^{109}Cd^{2+}$ with GSH and gel-filtration of the mixture on Sephadex G-15 yielded two major ^{109}Cd-labeled peaks: a low molecular weight peak *(LMW-Cd.GS)* and a high molecular weight peak *(HMW-Cd.GS)* (Figure 9A). When rechromatographed on Mono-Q, *LMW-Cd.GS* and *HMW-Cd.GS* eluted at 0 (Figure 9C) and 275 mM NaCl, respectively (Figure 9B). Of these two ^{109}Cd-labeled components, *HMW-Cd.GS* alone, underwent *YCF1*-dependent transport. MgATP-dependent, uncoupler-insensitive *HMW-*^{109}Cd.GS uptake by DTY165 membranes increased as a single Michaelian function of concentration (K_m, 39.1 ± 14.1 μM; V_{max}, 157.2 ± 60.7 nmol/mg/10 minutes) (Figure 10A). By contrast, uptake of *LMW-*^{109}Cd.GS by DTY165 membranes was negligible at all of the concentrations examined (Figure 10B). Vacuolar membranes from DTY167 cells transported neither *HMW-*^{109}Cd.GS nor *LMW-*^{109}Cd.GS (Figures 10A and 10B).

Bis(glutathionato)cadmium Is the Transport-Active Complex

The transport-active complex, *HMW-Cd.GS,* was identified as *bis*(glutathionato)cadmium ($Cd.GS_2$) by three criteria: (i) The average Cd:GS molar ratio of the transported species, estimated from the ^{109}Cd:^{3}H ratios of the *HMW-Cd.GS* peaks obtained after chromatography of reaction mixtures initially containing $^{109}Cd^{2+}$ and [^{3}H]GSH on Sephadex G-15 and Mono-Q were 0.44 ± 0.09 and 0.49 ± 0.17, respectively (Table 6). (ii) DTY165 membranes accumulated ^{109}Cd and [^{3}H]GS in a molar ratio of 0.49 ±0.01 when incubated in media containing *HMW-*^{109}Cd.[^{3}H]GS, MgATP and gramicidin-D (Table 6). (iii) The principal ion peak detected after MALD-MS of *HMW-Cd.GS* had an m/z ratio of 725.4 ± 0.7, consistent with the molecular weight of *bis*(glutathionato)cadmium (724.6 Da, Figure 11). The transport-inactive complex, *LMW-Cd.GS,* on the other hand, was tentatively identified as *mono*(glutathionato)cadmium on the basis of its smaller apparent molecular size (Figure 9A), failure to bind Mono-Q (Figure 9C) and Cd:GS ratio of 0.67 ± 0.04 and 0.86 ± 0.07 after chromatography on Sephadex G-15 and Mono-Q (Table 6), respectively.

While an m/z ratio of 725 for *HMW-Cd.GS* would be equally compatible with the transport of Cd.GSSG, this is refuted by two findings: (i) GSSG alone does not promote *YCF1*-dependent uptake (Table 5). (ii) The transport-active complex is probably a mercaptide. Pretreatment of *HMW-Cd.GS* with 2-mercaptoethanol inhibits MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by DTY165 membranes by more then 80% (Table 6) and *S*-methylation abolishes the stimulatory action of GSH (Table 5).

TABLE 6. Molar Cd:GS ratios of *LMW-Cd.GS* and *HMW-Cd.GS* complexes fractionated by Sephadex G-15 and Mono-Q chromatography (Figure 9) before and after MgATP-dependent, uncoupler-insensitive uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Cd:GS ratios were estimated from the ^{109}Cd:[^{3}H] radioisotope ratios of samples prepared from $^{109}CdSO_4$ and [^{3}H]GSH. *HMW-*^{109}Cd.[^{3}H]GS was pretreated with 2-mercaptoethanol (2-ME) by heating a 1:4 mixture of *HMW-*^{109}Cd.[^{3}H]GS with 2-ME at 60°C for 10 minutes before measuring $^{109}Cd^{2+}$ uptake. Uptake was measured using 50 μM concentrations (as Cd) of the complexes indicated in standard uptake medium containing 5 μM gramicidin-D. Values shown are means ± SE (n = 3-6).

FRACTION	^{109}Cd UPTAKE (*nmol/mg/10 min*)		MOLAR RATIO Cd:GS	
	DTY165	DTY167	BEFORE UPTAKE	AFTER UPTAKE
Sephadex G-15				
HMW-Cd.GS	-	-	0.44 ± 0.09	-
LMW-Cd.GS	-	-	0.67 ± 0.04	-
Mono-Q				
HMW-Cd.GS	66.3 ± 2.7	5.6 ± 2.6	0.49 ± 0.17	0.49 ± 0.01
LMW-Cd.GS	4.4 ± 0.8	3.9 ± 1.4	0.86 ± 0.07	-
After 2-ME				
HMW-Cd.GS	11.9 ± 2.4	4.4 ± 3.0	-	-

$Cd.GS_2$ Transport is Directly Energized by MgATP

Purification of $Cd.GS_2$ enabled the energy requirements of YCF1-dependent transport to be examined directly and confirmed that more than 83% of the MgATP-dependent, uncoupler-insensitive Cd^{2+} transport measured using DTY165 membranes was mediated by YCF1. Agents that dissipate both the ΔpH and $\Delta\Psi$ components of the H^+-electrochemical gradient established by the V-ATPase (FCCP, gramicidin-D) or directly inhibit the V-ATPase, itself (bafilomycin A_1), decreased MgATP-dependent Cd-GS_2 uptake by vacuolar membrane vesicles from DTY165 cells by 22% (Table 7). Ammonium chloride which abolishes ΔpH while leaving $\Delta\Psi$ unaffected, on the other hand, inhibited uptake by only 15% (Table 7). From these results and the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors alone (Table 7), Cd.GS2 uptake by wild type membranes is

inferred to proceed *via* a YCF1-dependent, MgATP-energized pathway that accounts for most of the transport measured and a YCF1-independent pathway, primarily driven by the H^+-gradient established by the V-ATPase, that makes a minor contribution to total uptake.

Table 7. Effects of uncouplers and V-ATPase inhibitors on uptake of *bis*(glutathionato)cadmium ($Cd.GS_2$) by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 50 μM purified $^{109}Cd.GS_2$. Bafilomycin A_1, FCCP, gramicidin-D and NH_4Cl were added at concentrations of 0.5 μM, 5 μM, 5 μM, and 1 mM, respectively. Values outside parentheses are means ± SE (n = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITION	$^{109}Cd.GS_2$ UPTAKE (*nmol/mg/10 minutes*)	
	DTY165	DTY167
Control	105.8 ± 12.4 (100)	17.3 ± 2.7 (100)
Gramicidin-D	77.8 ± 6.4 (73.5)	9.3 ± 2.0 (56.6)
FCCP	62.2 ± 11.4 (58.8)	10.2 ± 1.6 (59.0)
NH_4Cl	89.8 ± 8.2 (84.8)	10.0 ± 1.7 (57.8)
NH_4Cl+ gramicidin-D	69.8 ± 12.0 (66.O)	8.8 ± 2.2 (50.9)
Bafilomycin A_1	81.8 ± 6.0 (76.6)	12.8 ± 3.6 (74.0)
Bafilomycin A_1+ gramicidin-D	70.2 ± 12.2 (66.4)	7.2 ± 2.4 (41.6)

$Cd.GS_2$ Competes with DNP-GS for Transport

As would be predicted if $Cd.GS_2$ and the model organic GS-conjugate DNP-GS follow the same transport pathway, the K_i for inhibition of MgATP-dependent, uncoupler-insensitive Cd.GS2 uptake by DNP-GS (11.3 ± 2.1 μM; Figures 10A and 10C) coincided with the K_m for DNP-GS transport (14.1 ± 7.4 μM).

Pretreatment with Cd^{2+}or CDNB Increases *YCF1* Expression

RNase protection assays of *YCF1* expression in DTY165 cells and measurements of MgATP-dependent, uncoupler-insensitive $^{109}Cd.GS_2$ and [^{3}H]DNP-GS uptake by vacuolar membranes prepared from the same cells after 24 hour of growth in media containing $CdSO_4$ (200 μM) or the cytotoxic DNP-GS precursor, CDNB (150 μM), demonstrated a parallel increase in all three quantities. *YCF1*-

specific mRNA levels were increased by 1.9- and 2.5-fold by pretreatment of DTY165 cells with $CdSO_4$ and CDNB, respectively (Figure 12). The same pretreatments increased MgATP-dependent, uncoupler-insensitive $^{109}Cd.GS_2$ uptake into vacuolar membrane vesicles by 1.4- and 1.7-fold and [^{3}H]DNP-GS uptake by 1.6- and 2.8-fold (Figure 12).

These investigations provide the first indication of the mechanism by which *YCF1* confers Cd^{2+} resistance in *S. cerevisiae* and its relationship to the transport of organic GS-conjugates by demonstrating that the integral membrane protein encoded by this gene specifically catalyzes the MgATP-energized uptake of *bis*(glutathionato)cadmium by vacuolar membrane vesicles.

The codependence of Cd-GS_2 and organic GS-conjugate transport on *YCF1* is evident at multiple levels: (i) The *ycf1*Δ mutant strain, DTY167, is hypersensitive to Cd^{2+} and CDNB in the growth medium and both hypersensitivities are alleviated by transformation with plasmid-borne *YCF1*. (ii) Vacuolar membrane vesicles purified from DTY167 cells are grossly impaired for MgATP-energized, uncoupler-insensitive organic GS-conjugate and GSH-promoted Cd^{2+} uptake. (iii) $Cd.GS_2$, and organic GS-conjugates compete for the same uptake sites on YCF1. (iv) Factors that increase *YCF1* expression elicit a parallel increase in $Cd.GS_2$ and organic GS-conjugate transport. Thus, a number of ostensibly disparate observations, the strong association between cellular GSH levels and Cd^{2+} resistance (*e.g.*, Singhal *et al.*, 1987, *FASEB J.* 1:220-223), the markedly increased sensitivity of vacuole deficient *S. cerevisiae* strains to Cd^{2+} toxicity, and the coordinate regulation of the yeast *YCF1* and *GSH1* genes, the latter of which encodes γ-glutamylcysteine synthetase (Wemmie *et al.*, 1994, *supra*; Wu *et al.*, 1994, *supra*), are now explicable in terms of a model in which YCF1 catalyzes the GSH-dependent vacuolar sequestration of Cd^{2+}.

Further, at the biochemical level, YCF1 specifically catalyzes the transport of $Cd.GS_2$ as the data provided herein establish. In addition, at the cellular level, YCF1 confers resistance to and is induced by a spectrum of xenobiotics. Expression of *YCF1* is increased by exposure of cells to glutathione-conjugable

xenobiotics and Cd^{2+}. The close resemblance of YCF1 to MRP1, the capacity of YCF1 for both organic toxin and heavy metal transport, and its discovery in one of the most tractable and thoroughly molecularly characterized eukaryotes, *S. cerevisiae*, establishes that YCF1 is useful for manipulation of the transport of organic toxins and heavy metals in plants, mammals and yeast. Thus, according to the present invention, methods for overcoming, or at least diminishing, heavy metal contamination through bioremediation using native species or genetically engineered organisms are now possible.

Cloning of plant *MRP1/YCF1* homologs

As described herein, the data presented herein establish that *YCF1* encodes a protein functionally equivalent to human MRP1. There is next described the discovery that two plant genes, *AtMRP1* and *AtMRP2*, from *Arabidopsis* encode MRP1/YCF1 homologs.

To isolate genes likely involved in glutathione *S*-conjugate transport from *Arabidopsis thaliana*, degenerate PCR primers corresponding to appropriate portions of human *MRP1* (Cole *et al.*, 1992, *supra*) and *YCF1* (Szczypka *et al.*, 1994, *supra*) were designed. Four degenerate primers were synthesized but only two of these yielded amplification products of the appropriate size that hybridized with *MRP1* and *YCF1*. The sequences of the two primers were:
5'-GARAARGTIGGIATHGTIGGIMGIACIGGIGC-3'(MRP2) (SEQ ID NO:17) and 5'-TCCATDATIGTRTTIARICKTGIGC-3'(MRP4) (SEQ ID NO:18), where I = inosine, K = T or G, M = C or A and R = A or G. MRP2 corresponds to positions 1321-1331 and 1300-1310 in *MRP1* and *YCF1*, respectively; MRP4 corresponds to positions 1486-1494 and 1466-1474. Database searches confirmed that the sequences of the peptides specified by MRP2 and MRP4 were specific to *MRP1* and *YCF1* but not any other ABC transporter in GenBank database release 90 (Altschul *et al.*, 1990, *J. Mol. Biol.*, **215**: 403-410).

Degenerate PCR was performed using *Arabidopsis* genomic DNA as template. Amplification was for 45 cycles using the following thermal profile: 94°C

for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. A 0.6 kb PCR product was isolated, shown to hybridize strongly with a mixed probe encompassing the second NBF domain of *MRP1* and *YCF1*, and was cloned into pCRII vector (Invitrogen).

Sequence analysis verified that the deduced translation product of the *Arabidopsis* PCR product exhibited greatest similarity to YCF1 and MRP1 plus an unidentified 1.7 kb *Arabidopsis* EST (ATTS1246; Hofte *et al.*, 1993, *Plant J.*, 4: 1051-1061). In order to increase the likelihood of obtaining positive clones, a mixed probe consisting of the 0.6 kb PCR product and 1.6 kb EST was employed for further screens.

Eleven independent positive clones were obtained after screening approximately 3×10^5 plaques of a size-fractionated (3-6 kb) *Arabidopsis* cDNA library constructed in λZAPII (Kieber *et al.*, 1993, *Cell*, **72**: 427-441) with the mixed probe. Restriction mapping confirmed that all 11 isolates corresponded to the same gene. The longest of these inserts, a 3.5 kb insert, designated *AtMRP2*, was subcloned and sequenced (Figure 13).

Since this isolate of *AtMRP2* was estimated to be missing approximately 1.5 kb of the 5' sequence of the ORF (assuming that the complete ORF of *AtMRP2* is similar in size to the ORFs of human *MRP1* and yeast *YCF1*), 500 bp of the most 5' sequence of *AtMRP2* was used to probe two *Arabidopsis* bacterial artificial chromosome (BAC) libraries, UCD and TAMU (Choi *et al.*, 1995, *Weeds World*, **2**: 17-20) to isolate clones containing the missing sequence. This procedure yielded 8 BAC clones: U1L22, U8C12, U12A2, U23J22, U419, T9C22, T1B17 and T4K22. After digestion with *Hind*III, those fragments that hybridized with the 3.5 kb cDNA insert were introduced into pBluescript SK⁻ and sequenced. Two of these BAC clones (T1B17, T4K22) comprise a second *MPR1* plant homolog, designated *AtMRP1*, while the remainder (U1L22, U8C12, U12A2, U23J22, U419, T9C22) comprise *AtMRP2* (see below).

After establishing that an approximately 10 kb *Hind*III fragment from BAC clone U1L22 encompassed sequences identical to *AtMRP2*, a *Bgl*II restriction fragment comprising the first 3 kb of the BAC clone was used to rescreen

approximately 2 x 10^6 plaques from the *Arabidopsis* λZAPII cDNA library. Twenty six independent positive clones were obtained and the one containing the longest cDNA insert, 5.2 kb, was sequenced.

Sequence analysis demonstrated that the 5.2 kb cDNA was not identical to *AtMRP2* but instead a very closely related gene. Designated *AtMRP1* (Figure 16), the 5.2 kb cDNA was 84.3% and 88.2% identical to *AtMRP2* at the nucleotide and amino acid levels, respectively. Importantly, *AtMRP1* is a full-length cDNA.

Having determined the complete sequence of the *AtMRP1* cDNA, it was possible to identify the initiation codon of the *AtMRP2* genomic clone, design a specific 5'-UTR primer and amplify the remaining 5' end of *AtMRP2* to generate a full-length cDNA. Thus, full-length cDNAs encoding AtMRP2 and AtMRP1 (Figures 13 and 16, respectively) and genomic clones corresponding to *AtMRP2* and *AtMRP1* have been generated (Figure 14 and 17, respectively). The deduced amino acid sequences of AtMRP2 and AtMRP1 are presented in Figures 15 and 18, respectively.

Expression of *AtMRP1* in *Saccharomyces cerevisiae*

The experiments described below establish that AtMRP1 mediates the MgATP-dependent transport of GS-conjugates. The results of similar experiments on AtMRP2 demonstrate that this gene product has the same transport capability.

The data presented herein establishes that *YCF1* from *Saccharomyces cerevisiae* encodes a 1,515 amino acid ATP-binding cassette (ABC) transporter protein which localizes to the vacuolar membrane and catalyzes MgATP-dependent GS-conjugate transport. Membrane vesicles from wild type (DTY165) cells contain two pathways for transport of the model GS-conjugate, DNP-GS: an MgATP-dependent, uncoupler-insensitive pathway and an electrically driven pathway. Membranes from the mutant strains DTY167 and DTY168, harboring a deletion of the *YCF1* gene, are by contrast more than 90% impaired in MgATP-dependent, uncoupler-insensitive DNP-GS transport. Yeast strains lacking a functional *YCF1* gene therefore represent a model system for probing the GS-conjugate transport function of plant *YCF1/MRP1* homologs.

To test the transporter capacity of *AtMRP1* (the first clone for which a full-length cDNA was obtained) for conferring GS-conjugate transport, yeast strain DTY168 (disrupted for the *YCF1* gene) was transformed with an expression vector engineered to contain the coding sequence of *AtMRP1*. After selection of the transformants, membranes were prepared and assayed for MgATP-dependent, uncoupler-insensitive DNP-GS transport as described herein. The results establish that AtMRP1 catalyzes GS-conjugate transport in a manner indistinguishable from the vacuolar GS-X pump.

Construction of the expression vector

In order to constitutively express the *AtMRP1* gene in *S. cerevisiae*, a derivative of the yeast-*E. coli* shuttle vector, pYES2 (Invitrogen), was constructed. Essentially, the 831 bp *XbaI/NotI* fragment encompassing the 3-phosphoglycerate kinase (*PGK*) promoter of plasmid pFL61 (Minet et al., 1992, Plant J. 2:417-422) was inserted between the *SpeI/NotI* restriction sites of pYES2. In so doing, the galactose-inducible yeast *GAL1* promoter of pYES2 was replaced by the constitutive yeast *PGK* promoter, *pPGK*. This plasmid, designated pYES3, is otherwise identical to pYES2. The gene to be expressed is inserted into the multiple cloning site located between the *PGK* promoter and *CYC1* termination sequences.

Preliminary experiments had established that the 5' untranslated region (UTR) of the original *AtMRP1* cDNA isolate diminished expression of the open reading frame in yeast. Thus, to maximize expression, the 127 bp 5'-UTR of *AtMRP1* was removed. For this purpose, pBluescript SK$^-$-AtMRP1 was digested with *HpaI/Sna*BI to delete 3045 bp of the internal sequence. The remaining 5 kb fragment from this digest was gel-purified and self-ligated to generate truncated *AtMRP1* cDNA as a template for PCR. One hundred pmol of *AtMRP1*-Nco primer (5'-AAACCGGTGCGGCCGCCATGGGGTTTGAGCCGT-3') (SEQ ID NO:19) and 100 pmol of T3 primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO:20) were used to amplify a 2002 bp fragment of *AtMRP1* using Pfu DNA polymerase

(Stratagene). Amplification was for 30 cycles using the following thermal profile: 94°C for 15 seconds; 50°C for 15 seconds; and 72°C for 3.5 minutes.

The PCR product was gel-purified, digested with *Spe*I and cloned into the *EcoR*V/*Spe*I sites of pBluescript SK⁻ to generate pSK⁻ *AtMRP1*-Nco2. The 1227 bp *Sph*I/*Spe*I fragment of this construct was then exchanged with the 4363 bp *Sph*I/*Spe*I fragment of pBluescript pSK⁻ *AtMRP1* to generate pSK⁻ *AtMRP1*-Nco5. pYES-AtMRP1, lacking the 5' UTR, was constructed by digesting pSK⁻ *AtMRP1*-Nco5 with *Xho*I/*Spe*I to obtain a 5049 bp truncated *AtMRP1* gene fragment which was cloned into the *Xho*I/*Xba*I sites of pYES3. One kb of the 5' sequence of the *AtMRP1* insert of pYES3-*AtMRP1* was analyzed and was found to match exactly the sequence of the original cDNA clone.

Transformation of Yeast

S. cerevisiae strain DTY168 (MATα *his6*, *leu2*-3, -112, *ura3*-52 *ycf1*::*his*G) was transformed with pYES3-*AtMRP1* or empty vector lacking the *AtMRP1* insert (pYES3) by the LiOAc/PEG method (Giest et al., 1991, Yeast 7:253-263) and selected for uracil prototrophy by plating on AHC medium containing tryptophan (Kim et al., 1994, *supra*).

Isolation of membrane vesicles

For the preparation of membrane vesicles, 15 ml volumes of stationary phase cultures of the transformants were diluted into 1 L of fresh AHC medium and grown to an $OD_{600\ nm}$ of about 1.2. Membrane vesicles were purified as described herein and in Kim *et al.* (1995, *supra*).

Measurement of DNP-GS uptake

DNP-GS uptake was measured as described herein in 200 μl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of [^{3}H]DNP-GS (17.4 mCi/mmol). Gramicidin-D (uncoupler) was included to abolish the H^+-

electrochemical potential difference that would otherwise be established by the V-ATPase in media containing MgATP.

The results of this study

Membrane vesicles purified from pYES3-*AtMRP1*-transformed DTY168 cells exhibit an approximately 4-fold increase in MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by comparison with membrane vesicles purified from DTY168 cells transformed with empty vector (Figure 19). When measured at a DNP-GS concentration of 61.3 μM, the initial rates of uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed cells were 0.4 nmol/mg/minute and 0.1 nmol/mg/minute, respectively (Figure 19).

The concentration dependence and vanadate inhibitility of uptake verify direct participation of AtMRP1. MgATP-dependent, uncoupler-insensitive uptake by membrane vesicles purified from the pYES3-*AtMRP1* transformants increases as a single hyperbolic function of DNP-GS concentration to yield K_m and V_{max} values of 48.7 ± 15.4 μM and 6.0 ± 1.7 nmol/mg/10 minutes, respectively (Figure 19). pYES3-AtMRP1-dependent DNP-GS uptake decreases as a single exponential function of the concentration of the phosphoryl transition state analog vanadate, to yield an I_{50} of 8.3 ± 3.3 μM (Figure 20). By contrast, the apparent K_m for DNP-GS uptake by membrane vesicles purified from pYES3-transformed DTY168 cells is in excess of 500 μM and uptake is insensitive to vanadate.

On the basis of its sequence characteristics and the results of these experiments, *AtMRP1* encodes the vacuolar GS-*X* pump. The increases in uptake following the introduction of plasmid borne *AtMRP1* into yeast (*ca.* 4 nmol/mg/20 minutes) are commensurate with the rates of MgATP-dependent, uncoupler-insensitive DNP-GS uptake measured in vacuolar membrane vesicles purified from plant sources (2.3, 3.8, 18.2, 5.8, and 2.1 nmol/mg/20 minutes for *Arabidopsis* leaf, *Arabidopsis* root, *Beta vulgaris* storage root, *Vigna radiata* hypocotyl and *Zea mays* root, respectively) (Table III in Li *et al.*, 1995, *supra*). The K_m for DNP-GS transport by heterologously

expressed AtMRP1 is similar to that reported for the endogenous GS-*X* pump of plant vacuolar membranes (81.3 ± 41.8 μM, Li *et al.*, 1995, supra). The I_{50} for inhibition of AtMRP1-dependent DNP-GS transport by vanadate coincides with the I_{50} for inhibition of the endogenous vacuolar GS-X pump of plant cells (7.5 ± 3.9 μM, Li *et al.*, 1995, *supra*).

Having confirmed that the endogenous vacuolar *GS-X* pump of *S. cerevisiae* is lacking in the *ycf1*Δ mutant strains, DTY168 and DTY167 (Li *et al.*, 1995, *supra*), and in any case has a markedly lower K_m for DNP-GS and is 6 to 8-fold less sensitive to vanadate than the plant cognate, these findings establish that AtMRP1 *per se* is responsible for the MgATP-dependent, uncoupler-insensitive transport measured in these experiments. Given that heterologous expression of *AtMRP1* alone is sufficient for DNP-GS transport in DTY168 cells, it is concluded that one of the GS-*X* pumps of *Arabidopsis* has been cloned in its entirety.

Sequence comparisons of MRP1, cMOAT, YCF1, AtMRP1 and AtMRP2 with other members of the ABC transporter superfamily reveal two major subgroups. One group contains MRP1, cMOAT, YCF1, AtMRP1, AtMRP2 and the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA). The other group contains the MDRs, the major histocompatibility complex transporters and STE6. However, of all the ABC transporters defined to date, cMOAT, YCF1, AtMRP1 and AtMRP2 exhibit the closest resemblance to each other. Unlike the similarities between the GS-*X* pump subgroup, Lei/PgpA and CFTR, which center on the nucleotide binding folds (NBFs), the similarities between the GS-*X* pump members cMOAT, YCF1, AtMRP1 and AtMRP2 are found throughout the sequence. GS-*X* family members are 40-45% identical (60-65% similar) at the amino acid level, possess NBFs with an equivalent spacing of conserved residues and are colinear with respect to the location, extent and alteration of putative transmembrane spans and extramembrane domains. Two features of members of the GS-*X* pump family that distinguish them from other ABC transporters are their possession of a central truncated CFTR-like regulatory domain

rich in charged amino acid residues and an approximately 200 amino acid residue N-terminal extension.

A hydropathy aligment of AtMRP1, AtMRP2, YCF1, HmMRP1, and RtCMOAT is shown in Figure 21. Note the following: (i) The almost exact equivalence of AtMRP1 and AtMRP2 with respect to the alternation of hydrophobic and hydrophilic stretches. (ii) The close correspondence of AtMRP1 and AtMRP2 with all of the other members of the MRP1/YCF1/cMOAT subclass of ABC transporters in terms of the overall hydropathy profiles. (iii) The "signature" profile for the N-terminal 200 amino acid residues of all of the sequences shown, which is unique to the MRP1/YCF1/cMOAT subclass. Hydropathy was computed according to Kyte and Doolittle (1982, *J. Mol. Biol.* 46:105-132) over a running window of 15 amino acid residues. Hydrophobic stretches of sequence fall below the line and hydrophilic stretches fall above the line.

In Figure 22 there is depicted domain comparisons between AtMRP1, ScYCF1, HmMRP1, RtCMOAT, RbEBCR and HmCFTR. The domains indicated are the N-terminal extension (NH_2), first and second transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH). This comparison is also tabulated in Tables 8 and 9.

TABLE 8: Identity and similarity analysis of putative domains of AtMRP1 against AtMRP2 ScYCF1, HmMRP1, RtCMOAT, HmCFTR and RbEBCR. ScYCF1, *Saccharomyces cerevisiae* YCF1; HmMRP1, human MRP1; RtCMOAT, rat cMOAT; HmCFTR, human CFTR; RbEBCR, rabbit EBCR. The domains identified are N-terminal extension (NH_2), transmembrane segments 1 and 2 (TM1 and TM2, respectively), CFTR-like regulatory domain (R), nucleotide binding folds 1 and 2 (NBF1 and NBF2, respectively) and C-terminus (COOH). Similarity was calculated as described herein over the sequence segments indicated in Table 9.

SEQUENCE	DOMAIN	OVERALL	NH_2	TM1	NBF1
AtMRP2	*Identity*	87.0	74.4	90.4	92.1
	Similarity	93.7	85.2	96.1	96.7
ScYCF1	*Identity*	36.1	13.3	32.2	50.0

SEQUENCE	DOMAIN	OVERALL	NH_2	TM1	NBFI
	Similarity	55.4	32.9	52.6	75.0
HmMRP1	*Identity*	41.5	16.2	37.4	58.0
	Similarity	63.3	34.8	57.5	78.7
RtCMOAT	*Identity*	38.6	19.6	33.8	58.7
	Similarity	60.2	36.7	61.0	80.0
HmCFTR	*Identity*	29.2	0	22.8	40.7
	Similarity	55.1	0	47.8	62.0
RbEBCR	*Identity*	38.9	17.2	34.5	62.4
	Similarity	60.4	34.9	61.6	82.6

SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP2	*Identity*	80.5	86.9	91.3	89.4
	Similarity	89.8	94.2	96.5	94.4
ScYCF1	*Identity*	33.9	34.7	34.7	58.1
	Similarity	59.5	57.9	57.9	71.8
HmMRP1	*Identity*	31.6	31.9	61.9	48.3
	Similarity	50.4	56.3	72.8	69.0
RtCMOAT	*Identity*	33.9	34.4	60.7	50.0
	Similarity	50.0	58.8	75.7	67.2
HmCFTR	*Identity*	45.7	22.3	39.5	28.1
	Similarity	75.0	51.3	61.6	58.4
RbEBCR	*Identity*	35.6	34.1	61.9	43.0
	Similarity	51.7	59.1	74.0	62.7

TABLE 9. Positions and sizes of segments of sequence analyzed in Table 8.

SEQUENCE	DOMAIN	OVERALL	NH_2	TM1	NBF1
AtMRP1	*Position*		1-223	224-631	634-782
	Size	1622	223	407	148
AtMRP2	*Position*		1-223	224-631	634-782

SEQUENCE	DOMAIN	OVERALL	NH_2	TM1	NBF1
	Size	1622	223	407	148
ScYCF1	*Position*		1-210	211-645	646-787
	Size	1515	210	435	142
HmMRP1	*Position*		1-240	241-660	661-810
	Size	1531	240	420	150
RtCMOAT	*Position*		1-192	193-648	649-799
	Size	1540	192	456	151
HmCFTR	*Position*		0	1-440	441-590
	Size	1481	0	440	150
RbEBCR	*Position*		1-193	194-651	652-800
	Size	1562	193	458	149

SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP1	*Position*	783-900	901-1244	1245-1417	1418-1622
	Size	117	343	172	205
AtMRP2	*Position*	783-905	906-1249	1250-1422	1423-1622
	Size	122	343	172	200
ScYCF1	*Position*	788-936	937-1279	1280-1453	1454-1515
	Size	149	343	174	163
HmMRP1	*Position*	811-960	961-1300	1301-1473	1474-1531
	Size	150	340	173	59
RtcMOAT	*Position*	800-960	961-1302	1303-1476	1477-1541
	Size	161	342	174	65
HmCFTR	*Position*	591-847	848-1217	1218-1389	1390-1481
	Size	256	371	172	92
RbEBCR	*Position*	801-961	962-1304	1305-1477	1478-1562
	Size	161	343	173	86

As is apparent from the data presented above, there is significant homology between similar domains among AtMRP-related proteins. In particular, the N-terminal and R domains share significant homology among the AtMRP-related proteins tested. These data establish that in addition to primary sequence, the secondary structure of the molecule plays a significant role in GS-*X* pump function.

It should be appreciated that *AtMRP1* and *AtMRP2* constitute a family of genes in *Arabidopsis*, wherein various members of the family have different substrate specificities as demonstrated by the next set of experiments.

Substrate Preferences of AtMRP1 and AtMRP2

To examine the substrate preferences of AtMRP1 and AtMRP2, the following experiments were performed.

Isolation of *Bn*-NCC-1

[^{14}C]*Bn*-NCC-1 (33.3 mCi/mmol) was extracted from senescent cotyledons of rape (*Brassica napus*) and was purified by preparative HPLC (Krautler *et al.*, 1992, *Plant Physiol. Biochem.* 30:333-346). Determination of the purity of the final preparation by analytical HPLC and enumeration of concentration and specific radioactivity (33.3 mCi/mmol) were performed according to Hinder *et al.* (1966, *J. Biol. Chem.* 271:27233-27236). Unlabeled *Bn*-NCC-1 was isolated from fully senescent cotyledons of excised shoots that had been maintained in complete darkness for 1 week.

Measurement of transport

Cells were grown and vacuolar membrane-enriched vesicles were prepared as described (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635-). Uptake of [^{14}C]*Bn*-NCC-1, [^{3}H]C3G-GS, [^{3}H]DNP-GS, [^{3}H]GSSG, [^{14}C]metolachlor or [^{3}H]taurocholate was measured routinely in 200 μl reaction volumes containing membrane vesicles (10-20 μg protein), 3 mM ATP, 3 mM $MgSO_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine phosphate kinase, 50 mM KCl, 1mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated

concentrations of transport substrate. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol/3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate filters (pore size 0.45 μm). The filters were rinsed twice with wash medium, and the retained radioactivity was determined by liquid scintillation counting. Nonenergized uptake was estimated by the same procedure except that ATP was omitted from the uptake medium.

The effect of taurocholate on the release of [^{3}H]DNP-GS from membrane vesicles that had been allowed to mediate AtMRP2-dependent accumulation of this compound during a preceding uptake period was determined. This was accomplished by rapid depletion of ATP from the uptake medium using a hexokinase trap (glucose + ATP -> glucose-6-phosphate + ADP) and measurements of the decrease in vesicular radiolabel in the presence or absence of taurocholate. Membranes from DTY168/pYES3-AtMRP2 cells were incubated for 10 minutes in standard uptake medium containing 61.3 μM [^{3}H]DNP-GS after which time 200 mM glucose and 50 units/ml hexokinase (Type F-300 from baker's yeast) were added. After incubation for a further 2 minutes, taurocholate (50 μM) or Triton X-100 (9.01% v/v) was added and release of vesicular [^{3}H]DNP-GS was measured as described. Control samples were treated identically except that no additions were made after the initial 10 minute incubation period.

Substrate Preferences

The absence of AtMRP2-dependent transport from DTY168 and DTY168/pYES3 membranes and the selective inhibition of this system by micromolar concentrations of vanadate, established that AtMRP2-dependent transport may be measured in two ways. This may be accomplished by assessing the difference between the rates of MgATP-dependent, uncoupler-insenstitive uptake by DTY168/pYES3-AtMRP2 membranes by comparison with DTY168 or DTY168/pYES3 membranes, or by assessing the vanadate-sensitive component of MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP2 membranes. Because the results were

qualitatively and quantitatively similar whichever method was used, "AtMRP2-dependent" transport as used in this section refers to uptake which is measured as the increment consequent on transformation of DTY168 cells with pYES3-AtMRP2 *versus* pYES3.

Application of this methodology to vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2- *versus* pYES3-transformed DTY168 cells and expansion of the transport assays to measurements of the concentration dependence of [^{3}H]DNP-GS, [^{3}H]GSSG, [^{14}C]metolachlor-GS, [^{14}C]*Bn*-NCC-1 and [^{3}H]taurocholate uptake, demonstrated that the substrate preferences and maximal transport capacities of AtMRP2 and AtMRP1 differed markedly. While uptake of all of the GSH derivatives examined conformed to Michaelis-Menten kinetics, the V_{max} values for AtMRP2-dependent uptake were consistently serveralfold greater than those for AtMRP1-dependent uptake. The V_{max} values for AtMRP2-dependent uptake of [^{3}H]DNP-GS, [^{3}H]GSSG and [^{14}C]metolachlor-GS were 16.3 ± 3.1, 38.1 ± 3.2 and 136.0 ± 28.1 nmol/mg/10 min, respectively; the corresponding values for AtMRP1 were 8.2 ± 1.6 , 6.8 ± 1.1 and 17.5±5.2 nmol/mg/10 min. With the exception of [^{3}H]GSSG whose K_m for AtMRP1-dependent uptake (21.9.2 ± 58.3 μM) was three times greater than that for AtMRP2-dependent uptake (73.0 ± 15.1μM), the K_m values estimated for AtMRP2 and AtMRP1 were very similar (65.7±29.8 *versus* 63.6 ± 36.5 μM for metolachlor-GS).

Single concentration (50 μM) measurements of uptake of the glutathionated anthocyanin, cyanin-3-glucoside-GS (C3G-GS), demonstrated an approximately 6-fold greater capacity of AtMRP2 for transport of this compound (rate = 48.4 ± 2.2 nmol/mg/10 min) by comparison with AtMRP1 (rate = 7.9 ± 0.7 nmol/mg/10min).

In no case was MgATP-dependent, uncoupler-insensitive uptake of the unconjugated precursors of the GS-compounds, DNP, GSH, metolachlor and C3G detectable.

Neither AtMRP2 nor AtMRP1 catalyzed the uptake of [^{3}H]taurocholate. Transformation of DTY168 cells with either pYES3-AtMRP2 or pYES3-AtMRP1

conferred little or no increase in the capacity of vacuolar membrane-enriched vesicles for [^{3}H]taurocholate uptake over that measured with vesicles prepared from pYES3-transformed cells. The results of these experiments are presented in Table 10.

Table 10. Kinetic parameters for uncoupler-insensitive AtMRP1- and AtMRP2-dependent transport of GS-derivatives, *Bn*-NCC-1 and taurocholate.

	AtMRP1		AtMRP2	
Compound	K_m	V_{max}	K_m	V_{max}
DNP-GS	73.8 ± 18.8	8.2 ± 1.6	65.7 ± 29.8	16.3 ± 3.1
GSSG	219.2 ± 58.3	6.8 ± 1.1	73.0 ± 15.1	38.1 ± 3.2
Metolachlor-GS	63.6 ± 36.5	17.5 ± 5.2	75.1 ± 31.6	136.0 ± 28.1
Bn-NCC-1	------Linear------		15.2 ± 2.3	63.1 ± 2.5
Taurocholate	------Linear------		-----Linear------	

MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP1, DTY168/pYES3-AtMRP2 and DTY168/pYES3 membranes was measured as described herein. The K_m and V_{max} values were estimated by fitting the data to a single Michaelis-Menten function by nonlinear least squares analysis. Values shown are means ± SE.

The 2- to 8-fold greater capacity of AtMRP2 *versus* AtMRP1 for transport of the compounds examined was not attributable to differences in the levels of expression of their cDNAs from the *PGK* gene promoter of pYES3. Quantitative RT-PCR of equivalent amounts of total RNA extracted from DTY168/pYES3-AtMRP2 and DTY168/pYES3-AtMRP1 cells yielded similar levels of the 800 bp PCR

amplification product predicted from the sequences of the oligonucleotide primers used. Since neither amplification product was generated when PCR was performed without reverse transcription or when total RNA from DTY168/pYES3 cells was employed as template, contamination by genomic DNA and/or RT-PCR of transcripts other than those from *AtMRP2* or *AtMRP1*, respectively, was not responsible for the observed results.

Anomalous interactions between candidate transport substrates

Two critical properties of AtMRP2 were its capacity for the simultaneous transport of GS-conjugates and *Bn*-NCC-1 and its pronounced sensitivity to inhibition by taurocholate. Simultaneous measurements of [^{14}C]*Bn*-NCC-1 and [^{3}H]DNP-GS uptake by membrane vesicles purified from DTY168/pYES3-AtMRP2 cells revealed parallel accumulation of both compounds with little or no interference of the transport of one by the other. AtMRP2-dependent uptake of [^{14}C]*Bn*-NCC-1 at an extravesicular concentration equivalent to its K_m value (15μM,) was nearly three times less sensitive to DNP-GS than would be predicted if this GS-conjugate were a competitior. If DNP-GS were a simple competitive inhibitor such that its K_m value (66μM) approximated its K_i value for the inhibition of *BN*-NCC-1 uptake, 120 μM DNP-GS would be expected to inhibit [^{14}C]*Bn*-NCC-1 uptake by 48% but this was not observed. DNP-GS concentrations in excess of 120 μM decreased [^{14}C]*Bn*-NCC-1 uptake by less than 18%. Reciprocally, the concentration-dependence of AtMRP2-mediated [^{3}H]DNP-GS uptake was not affected appreciably by *Bn*-NCC-1. The K_m and V_{max} values for AtMRP2-dependent [^{3}H]DNP-GS uptake in the presence of 15 μM *Bn*-NCC-1 (80.5 ± 28.6 μM and 18.3 ± 1.6 nmol/mg/10/min) were similar to those measured in its absence.

Although neither AtMRP2 nor AtMRP1 transported taurocholate, AtMRP2-mediated transport was selectively inhibited by this compound. AtMRP1-dependent [^{3}H]DNP-GS uptake was relatively insensitive to taurocholate (I_{50} > 250 μM) but the uptake of both [^{3}H]DNP-GS and [^{14}C]*Bn*-NCC-1 by AtMRP2 was strongly inhibited ($I_{50(\text{DNP-GS uptake})}$ = 27 ± 1.3 μM; $I_{50(Bn\text{-NCC-1 uptake})}$ = 49.5 ± 0.3 μM).

Taurocholate at the concentrations employed appeared to exert is effect on AtMRP2-mediated transport by inhibiting pump activity directly rather than by increasing background membrane permeability and decreasing net influx by increasing passive DNP-GS efflux. Addition of taurocholate at a concentration (50 μM) sufficient to inhibit AtMRP2-dependent [^{3}H]DNP-GS uptake by 70% to DTY168/pYES3-AtMRP2 vesicles that had accumulated [^{3}H]DNP-GS for 10 minutes before arresting pump action by ATP depletion using a hexokinase trap, did not accelerate the efflux of intravesicular ^{3}H-label over that measured on vesicles subject to a hexokinase trap in the absence of taurocholate. Imposition of a hexokinase trap and addition of a concentration of detergent (Triton X-100; 0.01% v/v) known to permeate these membranes (Zhen *et al.*, 1997, *J. Biol. Chem.* 272:22340-22348), on the other hand, increased the rate and extent of release of the [^{3}H]DNP-GS accumulated during the preceding 10 minute uptake period by more than 3-fold *versus* DTY168/pYES3-AtMRP2 vesicles treated with hexokinase alone or hexokinase plus taurocholate.

The high capacity of AtMRP2 for the transport of large amphipathic anions other than GS-conjugates (i.e., *Bn*-NCC demonstrates that one pump can assume more than one of the several ABC transporter-like functions identified in plants to date. In the case of AtMRP2, this includes transport activity directed to a broad-range GS-conjugate pump and a chlorophyll metabolite pump. Thus, one the one hand, the high capacity of heterologously expressed AtMRP2, and to a lesser extent AtMRP1, for the transport of metolachlor-GS, and by extension GS-conjugates of other herbicides to glutathionation, is consistent with the molecular identification of transporters capable of removing these and related compounds from the cytosol. On the other hand, the high capacity of AtMRP2 for the transport of *Bn*-NCC is consistent with the identification of an element capable of contributing to the further metabolism and eventual removal of tetrapyrrole derivatives generated during leaf senescence from the cytosol.

Vacuolar uptake of glutathionated medicarpin by the glutathione conjugate pump

A key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. Accordingly, most studies of plant-pathogen interactions in the Leguminosae have centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, it is essential that they are accumulated in the plant in a site which is sequestered (*i.e.*, isolated) from the cytoplasm.

The following experiments describe uptake of free [^{3}H]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung bean (*Vigna radiata*). This uptake is slow and relatively insensitive to MgATP. However, after incubation with glutathione and a total glutathione-*S*-transferase preparation from maize (*Zea mays*), [^{3}H]medicarpin uptake occurs at a rate which is 8-fold faster in the presence, as opposed to the absence of MgATP. MgATP-dependent uptake of glutathione/glutathione-*S*-transferase pretreated [^{3}H]medicarpin is only slightly inhibited by uncoupler (gramicidin D), but is strongly inhibited by vanadate and the model glutathione-*S*-conjugate, *S*-(2,4-dinitrophenyl)glutathione. These results demonstrate that the MgATP-energized glutathione-conjugate pump identified herein in the membrane preparation is capable of high affinity, high capacity transport of glutathionated isoflavonoid phytoalexins. The experimental procedures and results of these experiments are now described.

Preparation of [^{3}H]medicarpin

[^{3}H]medicarpin was produced by base-catalyzed tritium exchange from ^3H_2O using unlabeled medicarpin isolated from fenugreek (*Trigonella foenumgraecum*) seedlings exposed to 3 mM $CuCl_2$.

GST purification and conjugation of medicarpin

Two-week old maize (*Zea mays*) B73N seedlings were grown under continuous light at 21°C. Twenty four hours prior to harvesting, the seedlings were exposed to a mild treatment with 2,4-dichlorophenoxyacetic acid and atrazine to stimulate GST expression (Timmerman, 1989, *Physiol. Plant* 77:465). Two-gram samples of root and shoot tissue were ground to homogeneity in 50 ml of 500 mM sodium phosphate buffer, pH 7.8 (Buffer A). The extract was centrifuged at 7,000 x g for 10 minutes at 4°C and the resulting supernatant was filtered through Miracloth and mixed with 2 ml of *S*-hexylglutathione-conjugated agarose beads (Sigma). After incubation for 5 minutes at 21°C, the beads were sedimented by centrifugation at 500 x g at 4°C. The supernatant was discarded and the beads were resuspended in 2.5 ml of prechilled Buffer A and centrifuged again. Bound GST was eluted by resuspension of the beads in 2 ml Buffer B (20 mM GSH, 500 mM sodium phosphate, pH 7.8) and incubation for 5 minutes at 21°C. The beads were sedimented by centrifugation at 500 x g and the supernatant was assayed for GST activity (Mannervick *et al.*, 1981, *Methods Enzymol.*, 77:231).

[^{3}H]medicarpin (0.5 µCi, 4.5 Ci/mol) was conjugated with GSH by incubation with 25 µl of total purified maize GSTs for 3 hours at 21°C in the dark. Control, unconjugated samples were prepared by mixing [^{3}H]medicarpin (0.5 µCi) with cold Buffer B and immediately freezing the mixture in liquid nitrogen.

Synthesis of S-(2,4-dinitrophenyl)glutathione (DNP-GS)

DNP-GS was synthesized from 1-chloro-2,4-dinitrobenzene and GSH by a modification of the enzymatic procedure of Kunst *et al.*, (1989, *Biochim. Biophys. Acta* **983**:123; Li *et al.*, 1995, *supra*).

Preparation of vacuolar membrane vesicles

Vacuolar membrane vesicles were purified from etiolated hypocotyls of *V. radiata* cv. Berken as described (Li *et al.*, *Plant Physiol.* **109**: 1257, Li *et al.*, 1995, *supra*).

Measurement of uptake

Unless otherwise indicated, [^{3}H]medicarpin or [^{3}H]medicarpin-GS uptake was measured at 25°C in 200 µl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 10 mM creatine phosphate, 16 U/ml creatine kinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol and 25 mM Tris-Mes buffer, pH 8.0. Uptake was initiated by the addition of 12 µl membrane vesicles (30-40 µg protein) and brief mixing of the samples on a vortex mixer. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted HA cellulose nitrate filters (pore diameter 0.45 µm). The filters were rinsed twice with a 1 ml ice-cold wash medium, air-dried and radioactivity was determined by liquid scintillation counting.

Protein estimations and source of commercial chemicals was as described herein.

Results

Appreciable MgATP-dependent uptake of [^{3}H]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung was dependent on preincubation of this compound with GSH and GSTs. Free [^{3}H]medicarpin incubated in the presence of GSH in the absence of GSTs was taken up at 16.7 ± 3.6 and 7.4 ± 1.3 nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25). In contrast, [^{3}H]medicarpin-GS synthesized by incubating [^{3}H]medicarpin with GSH in the presence of affinity-purified maize GSTs, was taken up at 81.0 ± 13.3 and 11.3 ±0.4 nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25).

MgATP-dependent [^{3}H]medicarpin-GS uptake was strongly inhibited by vanadate and DNP-GS but was relatively insensitive to uncouplers. Whereas inclusion of vanadate (10 µM) or DNP-GS (100 µM) in the assay medium inhibited [^{3}H]medicarpin uptake by more than 85%, addition of the ionophore, gramicidin D, diminished uptake by only 17% (Table 11).

Table 11. Effects of different inhibitors on [^{3}H]medicarpin-GS uptake by vacuolar membrane vesicles. [^{3}H]medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Gramicidin-D, vanadate and DNP-GS were added at concentrations of 5 μM, 10 μM and 100 μM, respectively. Values outside parentheses are means ± SE (n = 3); values inside parentheses are rates of uptake expressed as percentage of control.

TREATMENT	[^{3}H]Medicarpin-GS Uptake (*nmol/mg/10 minutes*)	
	+MgATP	-MgATP
Control	85.6 ± 13.3 (100)	16.7 ± 6.0 (100)
+ Gramicidin-D	71.2 ± 3.0 (83.2)	13.2 ± 2.1 (79.0)
+ Gramicidin-D + vanadate	12.9 ± 0.9 (15.1)	17.4 ± 0.5 (104.2)
+ Gramicidin-D + DNP-GS	11.7 ± 2.8 (13.7)	5.6 ± 3.1 (33.5)

MgATP-dependent, uncoupler-insensitive uptake increases as a single Michaelian function of [^{3}H]medicarpin-GS concentration to yield K_m and V_{max} values of 21.5 ± 15.5 μM and 77.8 ±23.3 nmol/mg/20 minutes, respectively (Figure 26).

Direct involvement of the GS-*X* pump in the accumulation of [^{3}H]medicarpin-GS by vacuolar membrane vesicles is therefore evident at three levels: (i) Glutathionation of medicarpin selectively increases MgATP-dependent uptake. MgATP-independent uptake is marginally affected by glutathionation but MgATP-dependent uptake is stimulated by approximately six-fold confirming that medicarpin-GS is the transported species and MgATP is the energy source. (ii) Uptake is directly energized by MgATP. The inability of uncoupler to markedly inhibit [^{3}H]medicarpin-GS uptake implies that the H^+-electrochemical gradient that would otherwise be established by the vacuolar H^+-ATPase in the presence of MgATP does not drive uptake. Rather, the pronounced inhibition of MgATP-dependent uptake exerted by vanadate agrees with the notion that GS-*X*-mediated uptake is strictly dependent on ATP hydrolysis and formation of a phosphoenzyme intermediate (Martinoia *et al.* 1993, *supra*; Li *et al.*, 1995, *supra*), (iii) [^{3}H]medicarpin-GS and the model GS-*X* pump substrate DNP-GS, whose transport has been exhaustively analyzed in this system as

described herein, compete for uptake indicating that both are transported by the same moiety.

The efficacy of medicarpin-GS as a substrate for the vacuolar GS-*X* pump is striking. Even though the K_m for medicarpin-GS uptake is undoubtedly an overestimate, since the yield from the conjugation reaction was not enumerated, it is nevertheless 2 to 25-fold lower than those estimated previously for DNP-GS, C3G-GS (80 and 46 μM in this system), glutathione-*S*-*N*-ethylmaleimide (500 μM) and metolachlor-GS (40-60 μM, barley vacuoles; Martinoia *et al.*, 1993, *supra*). Moreover, the capacity of the GS-*X* pump for medicarpin-GS uptake is high (V_{max} = 78 nmol/mg/20 minutes) versus DNP-GS (V_{max} = 12 nmol/mg/20 minutes) and comparable to that estimated for C3G-GS (V_{max} = 45 nmol/mg/minute). Thus, while maize anthocyanin was the first natural substrate shown to be vacuolarly sequestered through the concerted actions of cystolic GSTs and the vacuolar GS-*X* pump in plants (Marrs *et al.*, 1995, *Nature*, 375:397 and data contained herein), medicarpin, and presumably other isoflavonoid phytoalexins, is equally strong a candidate.

These data suggest that the GSTs which are induced following the hypersensitive response to avirulent fungal pathogens likely serve to facilitate the vacuolar storage of antimicrobial compounds in the healthy cells surrounding the lesion.

Transport of glutathionated anthocyanins and auxins by the vacuolar GS-*X* pump of plant cells

The data which are now described demonstrate that the vacuolar GS-*X* pumps of corn (*Zea mays*) roots and etiolated hypocotyls of mung bean (*Vigna radiata*) transport the anthocyanin cyanidin-3-glucoside (C3G), and the phytohormone, indole-3-acetic acid (IAA), after conjugation with glutathione. Whereas the unconjugated forms of these compounds undergo negligible uptake into vacuolar membrane vesicles, both C3G-GS and IAA-GS are subject to high rates of MgATP-dependent, uncoupler-insensitive uptake (Figure 27 and Table 12). IAA-GS and C3G-GS uptake approximates Michaelis-Menten kinetics to yield K_m values in the micromolar range

and V_{max} values 7- to 40-fold greater than those measured with the artificial transport substrate, DNP-GS (Table 12 and Li *et al.*, 1995, *supra*). Uptake of both conjugates is inhibited by DNP-GS and vanadate in a manner consistent with mediation by the GS-*X* pump (Figure 27 and Table 13). In contrast, glutathionated abscissic acid (ABA-GS) is a poor substrate for the GS-*X* pump: uptake is relatively slow and only saturates at high substrate concentrations (Figure 27 and Table 13).

Table 12. Summary of kinetic parameters for MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* and roots of *Z. mays*. Kinetic parameters (K_m, μM; V_{max}, *nmol/mg/10 min*) were computed from the data shown in Figure 27 by nonlinear least squares analysis. Values shown are means ± SE.

	C3G-GS		IAA-GS
PARAMETER	*V. radiata*	*Z. mays*	*V. radiata*
K_m	45.7 ± 14.0	39.5 ± 16.6	36.0 ± 16.7
V_{max}	45.3 ± 6.5	79.1 ± 14.7	17.7 ± 5.8

	IAA-GS	ABA-GS	
PARAMETER	*Z. mays*	*V. Radiata*	*Z. mays*
K_m	47.7 ± 19.6	>1000	128.8 ± 79.1
V_{max}	30.0 ± 4.9	22.9 ± 9.2	4.0 ± 1.4

It has been known for some time that the characteristic bronze coloration of *Bronze-2* (*bz2*) mutants is a consequence of the accumulation of cyanidin-3-glucoside in the cytosol. In wild type (*Bz2*) plants, anthocyanins are transported into the vacuole and become purple or red whereas in *bz2* plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a brown ("bronze") pigment. However, until the present invention, the exact molecular basis of this lesion was unknown. Since *Bz2* encodes a GST responsible for conjugating anthocyanin with GSH (Marrs *et al.*, 1995) and glutathionated anthocyanins are transported by the vacuolar GS-*X* pump, the

experiments described herein explain the bronze phenotype. Being defective in the glutathionation of anthocyanins, *bz2* mutants are unable to pump these pigments from the cytosol into the vacuole lumen; a conclusion borne out by the ability of the GS-*X* pump inhibitor, vanadate, to phenocopy the *bz2* lesion in wild type protoplasts and the efficacy of cyanidin-3-glucoside-GS as a substrate for the plant vacuolar GS-*X* pump *in vitro*, as the data presented herein establish.

The concept underlying the above-described experiments on phytohormones is that they may be subject to metabolic interconversions and compartmentation analogous to those deduced for anthocyanins. On the one hand, it is now established that C3G must be glutathionated before it can be transported into the vacuole. On the other hand, it is evident that most of the vacuolar anthocyanins of intact plants are not stored in this form. Instead, they are subject to long term storage as their malonyl derivatives. It is therefore apparent that while C3G-GS is a short-lived but necessary intermediate for vacuolar anthocyanin compartmentation, it is not the terminal product of this process. The experiments with auxins further illustrate this principle by demonstrating that IAA is susceptible to glutathionation and that the resultant IAA-GS conjugate is transported by the vacuolar GS-*X* pump in a MgATP-dependent, uncoupler-insensitive, vanadate-inhibitible manner. Thus, even though IAA-GS derivatives have not been detected *in planta,* this does not exclude the possibility that they are short-lived transport intermediates necessary for subsequent vacuolar processing of this class of compounds.

Table 13. Concentrations of DNP-GS and vanadate required to inhibit MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by 50% (I_{50} values) by vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* and roots of *Z. mays*. I_{50} values (μM) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential. ND, not determined.

	C3G-GS	IAA-GS	ABA-GS
COMPOUND	*V. radiata*	*Z. mays*	*V. radiata*
Vanadate	7.9	8.2	6.5
DNP-GS	103.5	112.4	124.2
	C3G-GS	IAA-GS	ABA-GS
COMPOUND	*Z. mays*	*V. radiata*	*Z. mays*
Vanadate	5.5	ND	>150
DNP-GS	109.8	ND	231.5

Generation of a Transgenic Plant Comprising a Transgene Encoding a GS-*X* Pump

To generate a transgenic plant comprising a gene encoding YCF1, the following experiments were performed. The binary vector pROK-*YCF1*, encoding wild type YCF1 was constructed. The sense orientation of the inserts with respect to the CaMV 35S promoter of pROK (Baulscombe *et al.*, 1986, *Nature* **321**:446-449) was confirmed and these constructs, as well as empty vector (pROK) controls, were transformed into *Agrobacterium* strain C58 by electroporation (Ausubel *et al.*, 1992, *Current Protocols in Molecular Biology*, pp 27-28).

Kanamycin-resistant *Agrobacterium* transformants were isolated, the integrity of the constructs in the bacterial recipient was established by PCR and *Arabidopsis* roots were inoculated with the transformants (Huang *et al.*, 1992, *Plant Mol. Biol.* **10**:372-384). The resulting rosette shoots generated on selective medium

were transferred to root-inducing medium for regeneration. Stable insertion of the sense strands and constitutive expression of *YCF1* and *YCF1::HA* was demonstrated in the kanamycin-resistant *Arabidopsis* transformants, by probing Southern blots with *YCF1* and pROK sequences and by Northern analyses, respectively.

An association between *YCF1* expression and altered xenobiotic resistance was tested by screening multiple T2 generation pROK-*YCF1*, pROK-*YCF1-HA* and pROK empty vector transformant lines for tolerance to cadmium salts and the GS-conjugable xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB). CDNB has three advantages for studies of this type: (i) It is an established plant toxin (Li *et al.*, 1995, *Plant Physiol.* **109**:117-185); (ii) The kinetics of transport of its glutathionated derivative, DNP-GS, as well characterized for YCF1 (Li *et al.*, 1996, *J. Biol. Chem.* 271:6509-6517) and the endogenous GS-X pump (Li *et al.*, 1995, *Plant. Physiol.* **107**:1257-1268; Li *et al.*, 1995, *Plant Physiol.* **109**:117-185). (iii) DNP-GS is the only known immediate metabolite of CDNB *in vivo* (Li *et al.*, 1995, *Plant Physiol.* **109**:117-185).

Methods similar to those described by Howden *et al.* (Howden *et al.*,1992, *Plant Physiol.* **99**:100-107; Howden *et al.*, 1995, *Plant Physiol.* **107**:1059-1066; Howden *et al.*, 1995, *Plant Physiol.* 107:1067-1073) were applied to the initial characterization of the transformants. T2 seeds were first sown in rows on Cd^{2+}-free and CDNB-free medium in Petri dishes standing on edge so that the roots grew vertically down the surface of the agar. Three to 4 days after germination, the seeds were transferred, again in rows, to media containing a range of $CdSO_4$ or CDNB concentrations. After rotating the Petri dishes though 180o and allowing growth for another 24-48 hours, the seedlings were scored for hook length. The results of this study are shown in Figure 28. It is evident from the data presented therein that transgenic *Arabiposis* plants comprising *YCF1* acquire increased resistance to cadmium salts and the organic cytotoxin, CDNB.

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

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Review

Plant ABC transporters

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Abstract

The ATP binding cassette (ABC) superfamily is a large, ubiquitous and diverse group of proteins, most of which mediate transport across biological membranes. ABC transporters have been shown to function not only as ATP-dependent pumps, but also as ion channels and channel regulators. Whilst members of this gene family have been extensively characterised in mammalian and microbial systems, the study of plant ABC transporters is a relatively new field of investigation. Sequences of over 20 plant ABC proteins have been published and include homologues of P-glycoprotein, MRP, PDR5 and organellar transporters. At present, functions have been assigned to a small proportion of these genes and only the MRP subclass has been extensively characterised. This review aims to summarise literature relevant to the study of plant ABC transporters, to review methods of cloning, to discuss the utility of yeast and mammalian systems as models and to speculate on possible roles of uncharacterised ABC transporters in plants.

Keywords: ABC transporter; P-glycoprotein; Multidrug resistance associated protein; PDR-5; Glutathione conjugate pump; Plant

1. Overview

The ATP binding cassette (ABC) superfamily is a large and diverse group of proteins, whose members mediate a wide range of transport functions. Most ABC proteins are primary pumps, which use the energy of ATP hydrolysis to drive transport, but some also modulate the activity of heterologous channels, or have intrinsic channel activity. A few have non-transport functions [1]. Over 100 ABC proteins have been identified to date, in taxa ranging from bacteria to humans: completion of the yeast and *Escherichia coli* genome sequences has revealed 29 and 79 ABC proteins, respectively [2,3], and the catalogue of representatives from multicellular eukaryotes continues to grow with the progress of targeted research and of sequencing projects. Substrates assigned to members of this large family of transporters include compounds as varied as peptides, sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic acids and glutathione conjugates [1,5]. This impressive list reflects not only the number and diversity of these transporters, but also their

Abbreviations: ABC, ATP binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; cMOAT, canalicular multispecific organic anion transporter; DPC, diphenylamine-2-carboxylic acid; GST, glutathione *S*-transferase; GS-X, glutathione conjugate; K-ATP, ATP-sensitive K^+ channel; KCO, potassium channel opener; MDR, multidrug resistance; MDR-P-gp, P-glycoprotein which mediates multidrug resistance; MRP, multidrug resistance associated protein; NBF, nucleotide binding fold; NTP, nucleotide triphosphate (ATP, CTP, GTP or TTP); P-gp, P-glycoprotein; SUR, sulphonylurea receptor; TMD, transmembrane domain

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A

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atpgp1 LSVPAGKTIA LVGSSGSGK. STVVSLIERF YDPNSGQVLL DGQDLKTLKL
humdr1 LKVQSGQTVA LVGNSGCGK. STTVQLMQRL YDPTEGMVSV DGQDIRTINV
atmrp1 LDIPLGSLVA VVGSTGEGKT SLISAMLGEL PARSDATVTL RGS.......
humrp1 FSIPEGALVA VVGQVGCGKL SLLSALLAEM .DKVEGHVAI KGS.......
hucftr FKIERGQLLA VAGSTGAGKT SLLMMIMGEL .EPSEGKIKH SGR.......
                      Walker A

atpgp1 RWLRQQIGLV SQEPALFATS IKENILLGRP DADQVEIEEA ARVANAHSFI
humdr1 RFLREIIGVV SQEPVLFATT IAENIRYGRE NVTMDEIEKA VKEANAYDFI
atmrp1 ......VAYV PQVSWIFNAT VRDNILFG.A PFDQEKYERV IDVTALQHDL
humrp1 ......VAYV PQQAWIQNDS LRENILFG.C QLEEPYYRSV IQACALLPDL
hucftr ......ISFC SQFSWIMPGT IKENIIFG.V SYDEYRYRSV IKACQLEEDI

atpgp1 IKLPDGFDTQ VGERGLQLSG GQKQRIAIAR AMLKNPAILL LDEATSALDS
humdr1 MKLPHKFDTL VGERGAQLSG GQKQRIAIAR ALVRNPKILL LDEATSALDT
atmrp1 ELLPGGDLTE IGERGVNISG GQKQRVSMAR AVYSNSDVCI LDEPLSALDA
humrp1 EILPSGDRTE IGEKGVNLSG GQKQRVSLAR AVYSNADIYL FDDPLSAVDA
hucftr SKFAEKDNIV LGEGGITLSG GQRARISLAR AVYKDADLYL LDSPFGYLDV
                                          Walker B

atpgp1 ESEKLV...Q EALDRFMIGR TTLIIAHRLS TIRKADLVAV LQQGSVSEIG 391-586
humdr1 ESEAVV...Q VALDKARKGR TTIVIAHRLS TVRNADVIAG FDDGVIVEKG 415-610
atmrp1 HVGQQVFEKC I..KRELGQT TRVLVTNQLH FLSQVDKILL VHEGTVKEEG 636-820
humrp1 HVGKHIFENV IGPKGMLKNK TRILVTHSMS YLPQVDVIIV MSGGKISEMG 666-850
hucftr LTEKEIFESC VC..KLMANK TRILVTSKME HLKKADKILI LNEGSSYFYG 446-628
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B

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atpgp1  AGKTLALVGP SGCGKSSVIS LIQRFYEPSS GRVMIDGKDI RKYNLKAIRK
humdr1  KGQTLALVGS SGCGKSTVVQ LLERFYDPLA GKVLLDGKEI KRLNVQWLRA
atmrp1  PMDKVGIVGR TGAGKSSLLN ALFRIVELEK GRILIDECDI GRFGLMDLRK
humrp1  GGEKVGIVGR TGAGKSSLTL GLFRINESAE GEIIIDGINI AKIGLHDLRF
hucftr  PGQRVGLLGR TGSGKSTLLS AFLRLLN.TE GEIQIDGVSW DSITLQQWRK
                Walker A

atpgp1  HIAIVPQEPC LFGTTIYENI AYGHEC..AT EAEIIQAATL ASAHKFISAL
humdr1  HLGIVSQEPI LFDCSIAENI AYGDNSRVVS QEEIVRAAKE ANIHAFIESL
atmrp1  VVGIIPQAPV LFSGTVRFNL ...DPFSEHN DADLWESLER AHLKDTIRRN
humrp1  KITIIPQDPV LFSGSLRMNL ...DPFSQYS DEEVWTSLEL AHLKDFVSAL
hucftr  AFGVIPQKVF IFSGTFRKNL ...DPYEQWS DQEIWKVADE VGLRSVIEQF


atpgp1  PEGYKTYVGE RGVQLSGGQK QRIAIARALV RKAEIMLLDE ATSALDAESE
humdr1  PNKYSTKVGD KGTQLSGGQK QRIAIARALV RQPHILLLDE ATSALDTESE
atmrp1  PLGLDAEVTE AGENFSVGQR QLLSLARALL RRSKILVLDE ATAAVDVRTD
humrp1  PDKLDHECAE GGENLSVGQR QLVCLARALL RKTKILVLDE ATAAVDLETD
hucftr  PGKLDFVLVD GGCVLSHGHK QLMCLARSVL SKAKILLLDE PSAHLDPVTY
                                          Walker B

atpgp1  RSVQEALDQA CSGRTSIVVA HRLSTIRNAH VIAVIDDGKV AEQGSHSHLL 1051-1248
humdr1  KVVQEALDKA REGRTCIVIA HRLSTIQNAD LIVVFQNGRV KEHGTHQQLL 1062-1252
atmrp1  VLIQKTIREE FKSCTMLIIA HRLNTIIDCD KVLVLDSGKV QEFSSPENLL 1263-1459
humrp1  DLIQSTIRTQ FEDCTVLTIA HRNLTIMDYT RVIVLDKGEI QEYGAPSDLL 1319-1515
hucftr  QIIRRTLKQA FADCTVILCE HRIEAMLECQ QFLVIEENKV RQYDSIQKLL 1236-1431
```

Fig. 1. Sequence alignment of selected ATP binding domains. (A) N-Terminal nucleotide binding domain. (B) C-Terminal nucleotide binding domain. Five ATP binding domains have been aligned to illustrate conserved motifs common to ABC transporters. Numbers at the bottom of the figure indicate the amino acid residues used in the alignment. Walker A and B motifs (common to all ATP binding proteins) are underlined. The ABC signature motifs are marked by double underlining. The accession numbers of sequences used to construct this figure are: human CFTR M28668 [7], human MRP L05628 [10], human P-gp1 M14758 [11], *Arabidopsis* MRP1 AF008124 [12], *Arabidopsis* P-gp1 E1339433 [13].

unique nature. Many ABC transporters are relatively specific, but others are able to handle several chemically dissimilar compounds, and for this reason are of considerable academic and practical interest [1,5].

The first ABC transporters to be extensively characterised were the so-called prokaryotic periplasmic permeases, involved in nutrient uptake by bacteria [6], but recently, much attention has focussed on members of the superfamily with clinical significance, including the cystic fibrosis transmembrane conductance regulator (CFTR [7]), sulphonylurea receptor (SUR [8]) and transporters from humans, parasites, yeast and bacteria which mediate multiple drug resistance (MDR [9]). In the past decade, ABC transporters have emerged as an important and fascinating group of proteins in plants. Before it is possible to review the plant literature, it is necessary to introduce some background information on the ABC superfamily which has been obtained from mammalian and microbial systems.

2. Structure of ABC proteins

2.1. Domain organisation

Typically, an ABC transporter contains two copies each of two structural units: a highly hydrophobic transmembrane domain (TMD), and a peripherally located ATP binding domain or nucleotide binding fold (NBF), which together are often necessary and sufficient to mediate transport. The TMD domains form the pathway via which the substrate crosses the membrane, and in some cases, have been shown to contribute to the substrate specificity. The NBFs are oriented towards the cytoplasmic side of the membrane and couple ATP hydrolysis to transport. Within the NBF is a conserved region of approx. 200 amino acids, consisting of the Walker A and B boxes separated by the ABC signature motif (Fig. 1). It is this signature motif which distinguishes ABC transporters from other NTP binding proteins, such as kinases, which also contain the Walker sequences [14,15]. Sequence homology over the whole gene can be negligible between different ABC transporters, but in the conserved areas of the NBF it is typically 30–40% between family members, and this has proved useful in the isolation of ABC genes by approaches such as PCR and hybridisation with degenerate nucleotides [16].

The organisation of ABC genes is almost as varied as their function [4,17]; representative examples of eukaryotic ABC genes are presented in Fig. 2. In prokaryotes, the different domains are commonly encoded as separate subunits, with the component genes of the ABC transporter arranged in a single operon [1,4]. However, there are many prokaryotic ABC genes in which two or more domains are fused to form a single polypeptide. ABC transporters of eukaryotic organelles are also expressed as separate subunits, which may reflect the endosymbiotic origin of plastids and mitochondria, but fusion of domains is more common in nuclear-encoded eukaryotic ABC genes. The most frequent arrangement is four domains fused in a single polypeptide, although the sequential order of domains may vary, for example: transmembrane domains precede the nucleotide

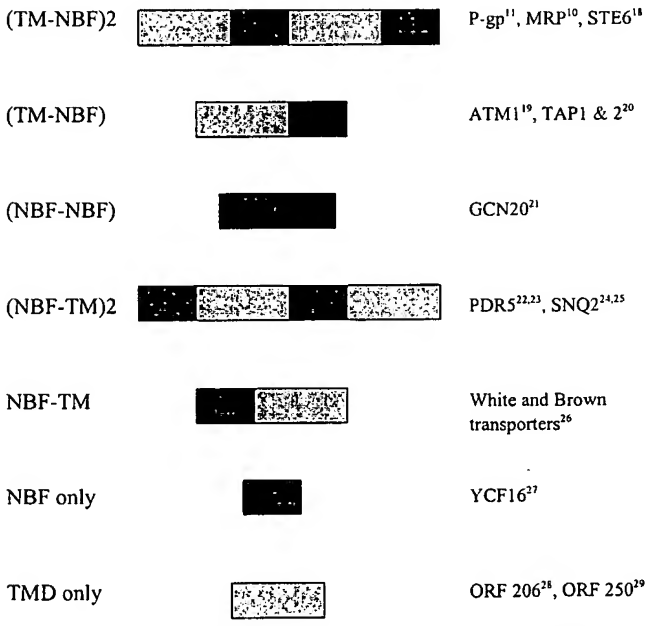

Fig. 2. Domain organisation of eukaryotic ATP binding cassette genes. The diagram shows the arrangement of domains in the RNA transcript. The transmembrane domains are represented as lightly shaded boxes, and the nucleotide binding domains are represented as dark shaded boxes. Examples for each arrangement are given. Note that *ycf16* and *ORF206, 250* are components of putative organellar ABC transporters, and require other subunits to form a functional transport protein. References [10,11,18–29] are given as superscripts above the gene names.

binding domains in MRP, STE 6 and P-glycoprotein (TMD-NBF-TMD-NBF), but PDR5 and SNQ2 exhibit the 'mirror' topology (NBF-TMD-NBF-TMD) (Fig. 2). Some eukaryotic ABC proteins, such as the HLA Class I antigen transporter (TAP) and the *Drosophila* white and brown gene products each consist of an ATP binding domain fused to a hydrophobic domain; these 'half-size' transporters are believed to function as dimers [20,26]. This flexibility of organisation is such that functional proteins can be obtained both by experimental separation and by fusion of domains [1], but whether four domains, either expressed as a single polypeptide or bound together to form an oligomer, represent the minimal structural unit for all ABC transporters is not yet confirmed (for a discussion, see [1]). Some members of the ABC superfamily which do not have transport functions, appear to have a different organisation e.g. OAB, which forms part of the ribonuclease L complex [30] and GCN20 which regulates protein kinase activity [21]. Additional domains occur in several ABC transporters; these may serve a regulatory function, for example, the CFTR R-domain [31,32]. In the case of the bacterial periplasmic permeases, an extra subunit serves to bind substrate and deliver it to the transporter [6]: these periplasmic binding proteins also have the ability to interact with membrane bound receptors as part of a signalling cascade [3,33].

2.2. Secondary structure

Given their varied domain organisation, it is difficult to make general statements about the secondary structure of ABC transporters [1]. As in the case of other membrane proteins, hydropathy analysis has indicated the likely disposition of transmembrane helices and hydrophilic loops, and biochemical studies to test theoretical models have been conducted for some proteins such as human MDR P-gp and MRP. P-gp is predicted to contain a tandem repeat of six transmembrane helices, each set followed by an ATP binding domain – the 'two-times-six paradigm'. Whilst this model has strong experimental support [34–36], the topology of MRP has been found to be significantly different [37]. Members of the MRP subfamily have a large, hydrophobic N-terminal extension, in addition to the core structure of two membrane associated and two ATP binding domains possessed by most ABC transporters. Secondary structure predictions combined with biochemical analyses suggest that MRP has 11 transmembrane spans in the N-terminal half of the protein and four or six in the C-terminal part [38,39]. Recent epitope insertion studies favour the latter model and indicate that the N-terminus is extracellular [40,41]. No hard evidence exists concerning the secondary structure of plant ABC transporters, but, given strikingly similar hydropathy profiles, parallels have been drawn from studies of homologues in other organisms (e.g. [5]).

2.3. Tertiary structure

In comparison to the abundance of biochemical and genetic data, little is known about the three-dimensional structure of ABC transporters. In a recent study, electron microscopy and image analysis of both reconstituted and detergent-solubilised human P-glycoprotein gave the first experimental insight into the 3D architecture of an ABC transporter [42]. The authors were able to propose a structural model which was in agreement with available biochemical and genetic data: the shape and size of the protein were consistent with the proposal that P-gp functions as a monomer containing two transmembrane domains, each consisting of six α-helices. Two 3 nm lobes exposed at the cytosolic face of the membrane were thought to correspond to the nucleotide binding domains. Three-dimensional reconstructions suggest that P-gp forms a large aqueous chamber within the membrane, open to the extracellular medium, but closed at the cytoplasmic face of the membrane, with an opening to the lipid phase [42]. The data were obtained in the absence of ATP; further information awaits the acquisition of data in the presence of ligands.

Crystallisation of membrane proteins is notoriously difficult, but attempts to crystallise ABC transporters or their separate domains are underway. In the absence of crystal data, an alternative approach has been to model ABC proteins on related, known structures, for example: folding patterns for the cytoplasmic NBDs of CFTR based on the mitochondrial F_1-ATPase [43,44] and aspartate aminotransferase [45], have been proposed, and are consistent with biochemical data. However, Hung et al. were re-

cently able to exploit the fact that ABC transporter domains are encoded as separate proteins in the histidine permease of *Salmonella typhimurium*, and determined the crystal structure for the ATP binding subunit, HisP [46]. Results from the more challenging task of crystallising an ABC transporter transmembrane domain are eagerly awaited; such information would greatly increase our understanding of the molecular mechanisms of transport.

3. ABC transporters and multidrug resistance: mechanistic implications

Cancer cell lines selected for resistance to specific cytotoxic drugs such as colchicine or doxorubicin often simultaneously acquire resistance to a number of structurally and functionally unrelated compounds – this phenomenon is known as multidrug resistance (MDR) and is of considerable clinical importance, since it renders tumours refractory to a number of chemotherapeutic agents [1]. A major advance in understanding MDR was the discovery that the human P-glycoprotein gene, *MDR1*, is overexpressed in a number of drug resistant cell lines. P-gp is thought to mediate ATP-dependent efflux of anticancer drugs such as *Vinca* alkaloids, anthracyclines, actinomycin D and taxol, thereby reducing their cytoplasmic accumulation. Since these compounds have little in common except for amphipathicity, a novel mechanism has been proposed to account from this unusual specificity – the 'flippase' model [47]. It has been proposed that P-gp binds an amphipathic molecule in the cytoplasmic leaflet of the plasma membrane and flips its polar group across the plasma membrane to deliver the molecule to the exocytoplasmic leaflet. The amphipathic molecule can now diffuse into the extracellular medium, effecting net removal from the cytoplasm. ATP is required to overcome the concentration gradient, which in this model is the differential substrate concentration between the two leaflets of the lipid bilayer. Not all members of the P-gp subfamily mediate drug transport, and perhaps ironically, support for the flippase model was obtained when the function of one such member was discovered. Mouse mdr2, although highly homologous to human MDR1 P-gp, does not pump drugs, but functions as a phosphatidylcholine translocase [48,49]. The equivalent protein in humans, MDR3 P-gp, also specifically translocates phosphatidylcholine, but MDR1 P-gp is a lipid translocase of broad specificity, which explains why it is able to handle multiple lipophilic drugs [50]. Structural data have offered further insight into the molecular basis of the flippase mechanism: three-dimensional reconstructions of electron microscopic data suggest that the aqueous chamber formed by P-gp has an opening to the lipid phase, allowing substrate access from the membrane to the pore translocation pathway (see above and [42]).

Other ABC transporters with low homology to P-gp are responsible for certain drug resistance phenotypes: for example, *MRP* was identified as being overexpressed in a multidrug resistant small cell lung cancer cell line, in which P-gp levels were normal [10]. Evidence from transfection experiments and studies of cell lines overexpressing *MRP* demonstrated that it is a glutathione conjugate transporter [51,52]. In addition to negatively charged glutathione conjugates, MRP also pumps lipophilic, neutral or mildly cationic cytotoxic drugs, in an unmodified form, with a substrate specificity which is overlapping, but distinct from that of P-gp [53,54]. The hydrophobic N-terminal extension of MRP has been postulated to play a special role in the interaction with anionic substrates [37], and to what extent structural and mechanistic models for P-gp can be applied to MRP in the light of this is yet to be determined.

ABC genes responsible for resistance phenotypes in the yeasts *Saccharomyces cerevisiae* and *Candida albicans* have also been well characterised in genetic and biochemical studies [2,55]. In *S. cerevisiae*, two linked networks of genes are responsible for the multidrug resistant phenotype: the PDR (pleiotropic drug resistance) and the YAP1 networks [56,57]. YAP1 is a leucine zipper transcription factor which mediates drug resistance through increased transcription of major facilitator superfamily (MFS) transporters, and also plays a role in resistance to oxidative stress. One of its targets is the vacuolar MRP homologue, YCF1 [58], which mediates resistance to Cd^{2+} [59] and acts as a transporter of glutathione conjugates and complexes [60,61]. The PDR network consists of two zinc finger transcriptional regulators Pdr1p and Pdr3p, which together activate the expres-

sion of the ABC transporters: PDR5, SNQ2 and YOR1. Whilst YOR1 is similar in organisation to human MRP [62], PDR5 and SNQ2 form a new class of ABC transporters, exhibiting a mirror topology with respect to MDR and MRP ([22–24,63–65]; Fig. 2). Despite a low level of sequence homology, and different organisation, they perform analogous roles to mammalian MDR P-gp, which has interesting structure/function implications. The precise specificities of Yor1p, Pdr5p, and Snq2p have been established in careful and elegant studies by Goffeau and co-workers, using a series of mutants in which combinations of the five PDR network genes are deleted [57]. The results of screening 359 toxins revealed that these three ABC transporters confer resistance to an extremely broad spectrum of compounds with distinct, but overlapping specificities. Not only antifungals, but also plant defence secondary metabolites were identified in the catalogue of transporter substrates. The existence of additional targets for the PDR1 and PDR3 transcriptional regulators was also evident from this work – possible candidates include several as yet uncharacterised ABC transporters identified in the yeast genome project [2,66]. Whilst comparisons of unicellular and multicellular organisms are not always appropriate [67], the amenability of yeast to genetic studies has revealed a complex and flexible defence system which may provide useful models for studies of detoxification in higher organisms.

4. The emergence of plant ABC transporters

In a 1992 review, only one plant ABC transporter was identified [1]. Currently, there are published reports of over 20 ABC genes from plants (Table 1), several others are represented in the EST databases, and various transport processes which may represent the activity of ABC transporters have been characterised. The role of the ABC superfamily in multidrug resistance phenomena has been an important factor in the development of plant transport research, since analogous roles for plant ABC genes in cross-resistance to herbicides have been postulated [13,89]. However, the diversity of roles in other taxa suggests that ABC transporters will certainly perform many other functions in plants. This potential, coupled with the possibility of employing highly conserved sequences from the NBF in the identification and isolation of genes, has led several groups to search for members of this gene family in plants. Two pioneering reports mark the start of plant ABC research in earnest: the isolation of an *MDR* homologue from *Arabidopsis thaliana* [13], and a biochemical study describing transport of glutathione conjugates into barley vacuoles, which could be ascribed to the activity of an MRP-like transporter [90]. Since then, reports of ABC transporter genes and activities have followed rapidly; these are discussed in detail below, and the reader is also referred to two excellent recent reviews on vacuolar conjugate transport [5,91].

5. MDR family

5.1. Molecular cloning of plant P-gp homologues

The first plant ABC transporter to be cloned was *AtPGP1*, an *MDR P-gp* homologue from *Arabidopsis* [13]. Degenerate oligonucleotide probes based on conserved regions of ABC transporters were used to screen an *Arabidopsis* genomic library, resulting in the isolation of a full-length genomic clone. The corresponding cDNA was subsequently isolated by PCR, permitting comparison of both coding sequence and gene structure with ABC transporter genes from other organisms. The *AtPGP1* clone exhibited similar intron structure to mammalian MDR genes, and encoded a protein with similar organisation of structural domains, suggesting that the P-gp subfamily evolved prior to the divergence of plants and animals [13]. Southern blot analysis of *AtPGP1* revealed the existence of a second, diverged member of the gene family; subsequently, a second homologue, *AtPGP2*, was cloned [13,16]. AtPGP1 and 2 share 44% amino acid identity, and are each 42% identical to human MDR1 [16]. Further similar, but non-identical *MDR*-like *Arabidopsis* genes have also been reported, though their sequences are not yet publicly available ([5]; Rea et al., unpublished results).

P-gp homologues have been identified in other plant species: screening of a potato stolon cDNA expression library with ^{35}S-labelled calmodulin re-

Table 1

a. Plant ABC proteins

Gene	Species	Functional information	Ref.
AtPGP1	*Arabidopsis*	role in light-dependent hypocotyl cell elongation	[13,68]
AtPGP2	*Arabidopsis*	?	[16]
PMDR1	potato	CaM binding site	[69]
HvMDR2	barley	?	[70]
AtMRP1	*Arabidopsis*	GS-X transport (DNP-GS, MOC-GS, C-3-G-GS)	[12]
AtMRP2	*Arabidopsis*	GS-X and chlorophyll catabolite transport (DNP-GS, *Bn*-NCC1), xenobiotic inducible	[71,72]
AtMRP3	*Arabidopsis*	GS-X and chlorophyll catabolite transport (DNP-GS, *Bn*-NCC1) confers Cd^{2+} resistance to *ycf1* mutant, xenobiotic inducible	[73,74]
AtMRP4	*Arabidopsis*	xenobiotic inducible	[75]
AtMRP5	*Arabidopsis*	GS-X transport	[76]
TaMRP1	wheat	xenobiotic inducible	[77]
TUR2	*Spirodela*	*PDR5* homologue, transcriptional regulation by hormones and stress	[78]
AtTUR2	*Arabidopsis*	*PDR5* homologue	[78]
SNQ2 homologue	alfalfa	induced in somatic embryogenesis	[79]
GCN20 homologues	rice, *Arabidopsis*	regulation of kinase activity?	[21]

b. Organellar ABC proteins

Gene/ORF	Species	Function	Mit/c.plast?	Ref.
orf277	*Marchantia*	haem transport, *helB* homologue	M	[80]
orf206	*Oenothera*, *Arabidopsis*, carrot, tomato	haem transport, *helB* homologue	M	[28,81]
?	*Marchantia*	haem transport, *helC* homologue	M	[80]
orf240	wheat, rice	haem transport, *helC* homologue	M	[29,82]
orf250	*Oenothera*, *Arabidopsis*, carrot	haem transport, *helC* homologue	M	[83]
ycf16	*Odontella*	located in stroma	C	[27]
Aorf2	*Antithamnion* sp.	?	C	[84]
orf257	*Galdiera sulphuraria*	?	C	[85]
mbpX	*Marchantia*	?	C	[86,87]
mbpY	*Marchantia*	forms complex with *mbpX*?	C	[88]

sulted in the isolation of *PMDR1*, which is 86% identical to *AtPGP1* and 41% identical to human *MDR1* P-gp [69]. The cloning method employed suggests an intriguing link to calcium-dependent signalling pathways, but the function of *PMDR1* has not yet been reported; the authors tentatively propose a role in tuberisation, based on high levels of expression in stolon tips [69]. A P-gp homologue has also been cloned from barley, using a semi-nested degenerate PCR approach [70]. Two PCR products with homology to ABC transporters (*HvMDR1* and *2*) were originally identified, and a full-length cDNA isolated for *HvMDR2*. Examination of the nucleotide sequence indicates that *HvMDR1* represents a separate putative ABC transporter, and is not a fragment of *HvMDR2*. The predicted amino acid sequence of *HvMDR2* is 43% identical to both *AtPGP1* and *2*, and 38% identical to human *MDR1*, suggesting that it is a novel P-gp homologue from plants. *HvMDR2* is expressed at very low levels in roots and shoots, and Southern analysis of different barley cultivars suggests that there is a small family of related genes in this species, with some polymorphism between cultivars. The function of *HvMDR2* has not yet been determined.

Comparison of these plant *P-gp* homologues reveals many similarities to other *MDR* genes: in primary sequence, predicted topology and domain organisation. However, the proteins encoded by the plant genes are significantly smaller than their mam-

malian counterparts: between 134 and 144 kDa, compared with 170–180 kDa for human P-gp [1], and whilst all plant *P-gp* homologues isolated to date have potential sites for N-linked glycosylation, none of these correspond to the glycosylation site of human P-gp in the first predicted extracellular loop [13,69,70]. Whether the plant transporters are glycosylated remains to be determined experimentally.

5.2. Functions of MDR homologues

The first and, to date, only, functional characterisation of a plant P-gp was achieved with transgenic plants. In this study, Dudler and coworkers manipulated the expression of *AtPGP1* in transgenic *Arabidopsis* plants using sense and antisense constructs, and their results demonstrated the involvement of AtPGP1 in hypocotyl cell elongation in the light [68]. The authors propose that AtPGP1 is involved in the export of a signal compound, possibly a peptide hormone, from the shoot apical region. In agreement with this hypothesis, expression of *AtPGP1* was analysed using reporter gene constructs and in situ hybridisation, and found to be located in apical regions of shoots and roots. Overexpression of *AtPGP1* in transgenic plants facilitated its subcellular localisation: c-myc tagged protein was visualised in the plasma membrane using immunofluorescence confocal microscopy, and results were confirmed by Western blotting of membrane fractions. A plasma membrane location for AtPGP1 is also consistent with a signalling/export role.

Since it is clear that plants have at least a small gene family of MDR-like transporters, the question of whether they perform functions analogous to those of homologues in other organisms arises. Determining the function of cloned ABC transporters is problematic: where available, full-length cDNAs can be expressed in heterologous systems, such as the yeast *S. cerevisiae*, but without additional information to guide experiments, systematically testing the list of possible substrates is prohibitive. Examination of the ABC transporter literature suggests a range of putative functions: for example, yeast STE 6 transports peptides [18], mammalian MDR2 functions as a phospholipid translocator [48,49], and P-gp/MDR1 exports cytotoxic drugs from the cells and acts as a channel regulator [92] – all these are plausible functions for P-gp homologues in plants. Moreover, the fact that plant secondary products such as vincristine and taxol are often substrates for, or inhibitors of, MDR proteins suggests a role of plant P-gp in synthesis and compartmentation of these compounds. It is not possible to predict substrate based on primary structure/sequence homology: the products of even closely related genes can have markedly different functions [1] and site-directed mutagenesis of many types of transport protein has shown that single amino acid changes can radically alter substrate specificity. Therefore, for transporters isolated without reference to their function, other approaches are needed: for example, a transgenic strategy first successfully identified the function of murine MDR2. Here, plant scientists have the advantage that generation of transgenic plants is relatively straightforward in many species, and the opportunity for generating tagged mutants exists for model plants such as *Arabidopsis*, petunia and maize [93]. However, phenotypes of antisense plants may not be immediately obvious, and may only be evident under specific conditions, such as stress, or at specific developmental stages.

6. MRP subfamily

Whilst the first higher plant ABC transporter to be cloned was a member of the MDR subfamily, investigations of the role of vacuolar transport in xenobiotic detoxification, together with important insights from ABC transporters implicated in heavy metal transport and drug resistance have led to the identification and characterisation of plant members of the MRP subclass [5]. Progress in characterising the roles of MRP in plants has been rapid, since the isolation of *MRP* genes has explained and extended results from detailed biochemical studies.

6.1. Detoxification: biochemical studies

The detoxification of lipophilic xenobiotics such as herbicides is a multistage process, commonly comprising activation (phase I), conjugation (phase II) and compartmentalisation (phase III) of a toxic compound [94,95]. Phases I and II are relatively well characterised in plants: activation may involve hy-

drolysis by esterases or amidases, but more commonly is an oxidation reaction catalysed by a cytochrome *P*-450 [95]. Often these reactions result in products of increased toxicity, but they serve to generate functional groups for the protective conjugation reactions of phase II. In phase II, the xenobiotic or its activated metabolite is covalently linked by a transferase enzyme to an endogenous, hydrophilic substance: glucose, glutathione or malonate in plants, and glutathione, glucuronate or sulphate in animals. These inactive, water-soluble conjugates are then transported from the cytoplasm in phase III. In animals, conjugates are excreted from the cell across the PM by a specific ATPase [96–98], but in plants, which have no excretion system, they are thought to be deposited in the vacuole, and may be further metabolised, eventually appearing as 'bound residues' in the extracellular matrix [99].

In 1993, Martinoia and coworkers demonstrated that intact vacuoles isolated from barley mesophyll mediated MgATP-dependent accumulation of glutathione conjugates [90]. Both the model substrate, *N*-ethylmaleimide-GS (NEM-GS) and a glutathione conjugate of the herbicide, metolachlor (metolachlor-GS) were investigated. Uptake was driven by MgATP, but not by non-hydrolysable ATP analogues, or by PPi. Transport of NEM-GS and metolachlor-GS was sensitive to vanadate, but unaffected by inhibitors of the vacuolar H^+-ATPase, and chemicals which collapse the tonoplast proton gradient, indicating that uptake of glutathione conjugates into the vacuole is mediated by a specific ATPase, and not by a secondary active process. Oxidised glutathione (GSSG) was also shown to be a substrate for this transporter, whereas reduced glutathione (GSH) was not [100]. The characteristics of this activity were strikingly similar to the ATPase in the canalicular membrane of liver, which exports glutathione conjugates and GSSH into the extracellular medium [101], and this important finding provided the first experimental evidence for the identity and location of the phase III transport step in plants.

Subsequently, confirmation of this phenomenon was obtained for other systems: Rea and co-workers demonstrated MgATP-energised transport of the model conjugate dinitrophenol-GS (DNP-GS) by vacuolar membrane vesicles from *Arabidopsis*, beet, maize and mung bean [102]. Interestingly, they also reported the partial sensitivity of DNP-GS transport activity to P-glycoprotein inhibitors, vinblastine and verapamil [102], in contradiction to Blake-Kalff and Coleman, who did not detect any notable effect of these compounds on NEM-GS uptake into barley vacuoles [103].

Thus, a substantial body of evidence suggested the participation of a glutathione conjugate (GS-X) transporter in the detoxification of xenobiotics. Evidence for vacuolar accumulation and further processing of herbicide-GS conjugates was obtained by Wolf et al., who demonstrated rapid accumulation of alachlor-GS in barley vacuoles, and characterised a carboxypeptidase which catalyses the first step in conjugate degradation [104]. Operation of the detoxification pathway in vivo, from conjugation to vacuolar sequestration, was elegantly confirmed by Coleman et al. using a monochlorobimane-based fluorescent assay in whole maize and carrot cells [105]. Further evidence that GS-X transport is part of an integrated detoxification pathway came from studies with herbicide safeners and xenobiotics. Safeners are a group of chemically diverse compounds which increase the tolerance of monocot crops to specific herbicides [106]. Gaillard et al. found that the cereal safener, cloquintocet mexyl, doubled the vacuolar transport activity for both glutathione and glucoside conjugates of herbicides in barley [107]; glutathione *S*-transferase (GST) activity was also increased by the treatment. Similarly, Li et al. found that pretreatment of mung bean seedlings with model GST substrate, 1-chloro-2,4-dinitrobenzene, increased the activity of DNP-GS transport in tonoplast vesicles [108]. In both studies, application of the exogenous compound increased the V_{max}, but did not affect the K_m, suggesting that the higher activity was due to increased expression of the transporter and not altered affinity for substrate [107,108].

6.2. Molecular cloning of GS-X transporters

The seminal paper of Martinoia and the reports that followed from the groups of Rea and Coleman provided strong evidence for an *MRP* homologue as the candidate gene encoding the plant vacuolar glutathione conjugate transporter [90,102,103,108]. *MRP* was originally isolated from a drug resistant small cell lung cancer line [10], and a related gene,

the canalicular multispecific organic anion transporter, *cMOAT*, has since been identified [109]. Biochemical studies with membrane vesicles suggest that an export ATPase for glutathione conjugates resides at the plasma membrane of several mammalian cell types [101], and functional studies demonstrate that *MRP1* [51,52] and *cMOAT* [97,109] are responsible for these activities. Moreover, the yeast cadmium factor, *YCF1*, an *MRP* homologue isolated by its ability to mediate cadmium resistance [59], was shown to be a vacuolar glutathione *S*-conjugate pump [60]. Functional complementation of a *ycf1* mutant with human *MRP1* lent further support to the importance of this ABC subclass in GS-X transport [110].

Thus began the search for *MRP* homologues in plants. Several *Arabidopsis* expressed sequence tags (ESTs) were identified as putative *MRP* homologues [73,95], and Tommasini et al. demonstrated that transcripts corresponding to certain *MRP*-like ESTs were up-regulated in response to xenobiotic treatment, in agreement with a probable role in detoxification [73]. The genes corresponding to these ESTs have now been cloned: *AtMRP1* and *2* as both full-length genomic and cDNA clones [12,71,72], and *AtMRP3* and *4* as cDNA and genomic clones respectively [73–75]. Identification of the yeast MRP homologue, YCF1, as a GS-X pump, and the availability of a *ycf1* deletion mutant DTY167, provided the intellectual basis and genetic background for the heterologous expression of plant MRP homologues, and hence *AtMRP 1–3* were characterised in yeast. All three genes encode glutathione conjugate transporters, with similar characteristics to GS-X uptake activities previously studied in tonoplast vesicles and isolated vacuoles [12,71,74]. There are significant differences between the isoforms, for example: the overall GS-X transport capacity of AtMRP2 greatly exceeds that of AtMRP1 [71], and AtMRP3, but not AtMRP1 or AtMRP2, was able to alleviate the Cd^{2+} sensitivity of ycf1 [71,74]. YCF1 has been shown to mediate Cd^{2+} resistance by vacuolar sequestration of $(Cd.GS)_2$ [61], but human *MRP1*, which also rescues the *ycf1* mutant, does not [110]. It is not currently clear whether AtMRP3 is competent in $(Cd.GS)_2$ transport [5,74].

Recently, a further *Arabidopsis* MRP homologue (*AtMRP5*) was cloned, and also shown to encode a glutathione conjugate transporter ([76]; N. Gaedeke, B. Müller-Röber, pers. comm.). A partial *MRP* homologue (*TaMRP1*) has also been isolated from wheat, in a screen for herbicide safener-induced genes [77]. The wheat gene bears closest sequence similarity to *AtMRP4*, which is consistent with the finding that *AtMRP4* also exhibits a transcriptional response to herbicide safeners [75]. *AtMRP4* and *TaMRP1* have yet to be characterised functionally.

6.3. Other substrates for MRP?

The ability of MRP to transport glutathione conjugates and the inducibility of MRP isoforms by herbicide safeners argues strongly for a role in herbicide metabolism. However, the application of herbicides is a recent event in evolutionary history [111], and therefore postdates the emergence of MRP, which appears to have evolved prior to the divergence of plants and animals. Since *Arabidopsis* has a small family of MRP genes encoding transporters with different kinetic properties, this raises the question of alternative/ancestral functions for MRP in plants. All plant MRPs characterised to date mediate GS-X transport, but few natural glutathionylated compounds have been demonstrated convincingly in plants. This may reflect low steady-state levels, due to further processing of glutathione conjugates once they have been sequestered in the vacuole: a vacuolar carboxypeptidase which cleaves the glycine residue from metolachlor-GS has been characterised [104], and further steps leading to the deposition of bound residues can be postulated [95,99]. However, an alternative explanation is that non-glutathionylated compounds also serve as MRP substrates. This is indeed the case for human MRP isoforms: substrates for cMOAT include bile acids [112], glucuronides [113] and unconjugated organic acids [114], and MRP1 transports glucuronate and sulphate conjugates in addition to glutathione conjugates [113, 115]. Similarly, a number of vacuolar transport activities which may be attributable to MRP have recently been characterised, and are summarised below.

6.3.1. Chlorophyll catabolites

During leaf senescence, the porphyrin moiety of chlorophyll is cleaved into linear tetrapyrroles, which

are eventually deposited in the vacuoles of mesophyll cells [116]. ATP-dependent uptake of these chlorophyll breakdown products into isolated barley vacuoles has been demonstrated by Hinder et al., and showed striking similarities with GS-X transport [117]. Following cDNA cloning of *Arabidopsis* MRP genes, yeast expression studies showed that AtMRP2 and 3 transport not only GS-X, but also chlorophyll catabolites such as *Bn*-NCC-1 [71,74]. Whilst *Bn*-NCC-1 is not glutathionylated, it is conjugated to malonate, and it is possible that malonylation may also serve to 'tag' compounds for sequestration in the vacuole, the action of a malonyltransferase playing an analogous role to that of glutathione *S*-transferases [118]. Competition experiments employing isolated vacuoles suggested that GS-X and *Bn*-NCC1 are sequestered by distinct transporters, but the heterologous expression experiments provide unequivocal proof that a single transporter is competent in the transport of both substrates. However, *AtMRP2* is not necessarily an orthologue of the barley transporter, since their K_m values for *Bn*-NCC1 differ by 10-fold [71]. Interestingly, DNP-GS and *Bn*-NCC1 did not compete for uptake when supplied simultaneously to tonoplast vesicles from yeast expressing *AtMRP2*, but were accumulated to levels comparable to those observed when only a single substrate was supplied. This unusual kinetic observation has important mechanistic implications, suggesting the presence of two distinct functional domains which operate independently in AtMRP2. A model accounting for all the kinetic data is developed in [71], and discussed further in [5]. It will be informative to determine whether dual transport can be reconciled with the flippase model proposed for MDR-type transporters.

6.3.2. Glucosides and glucuronides

Studies with isolated barley vacuoles indicate that different energisation mechanisms drive the uptake of flavonoid glucosides and herbicide glucosides [119]. The endogenous flavonoid glucoside, isovitexin, was taken up via a ΔpH, V-ATPase dependent mechanism, whereas hydroxyprimisulphuron-glucoside was transported by a vanadate-sensitive pump [119]. Induction of hydroxyprimisulphuron-glucoside transport activity by herbicide safeners [107], and induction of *AtMRP3* by primisulphuron [73] lend further support to the hypothesis that an MRP could be implicated in xenobiotic-glucoside transport, although inducing compounds do not necessarily serve as substrates. Similarly, glucuronides, which are generally rare in plants, but are abundant endogenous conjugates in rye, were shown to be transported by an MRP-like pump in isolated rye vacuoles [120].

6.3.3. Taurocholate

At the time of the discovery of the glutathione conjugate ATPase, a second vacuolar ATP-dependent anion transporter, which functioned as a taurocholate pump was identified in liver [121–123]. Perhaps surprisingly, Hörtensteiner et al. were able to demonstrate a similar activity in barley vacuoles [124]. Since bile acids, such as taurocholate, do not occur naturally in plants, the plant taurocholate transport activity must represent the operation of a transporter with an unknown, probably bulky, anionic substrate. As for the transport of glutathione conjugates, uptake of cholate conjugates, taurocholate and glycocholate, was ATP-dependent, inhibited by vanadate, but not by bafilomycin and could not be supported by non-hydrolysable ATP analogues, suggesting a primary active process. NEM-GS and taurocholate transport could be distinguished by their substrate specificity, response to alternative nucleotides and sensitivity to different inhibitors [103,124], and glutathione conjugates stimulated, rather than inhibited taurocholate transport [124]. It was therefore proposed that taurocholate and GS-X transport activities represented distinct transporters [124]. However, Blake-Kalff and Coleman found that taurocholate inhibited the uptake of NEM-SG into barley vacuoles [103]. The ability of vacuoles to maintain a proton gradient in the presence of taurocholate was verified by quinacrine fluorescence quenching, and taurocholate was supplied at a concentration below its critical micelle concentration, thereby eliminating inhibition due to detergent effects. In accordance with these observations, neither AtMRP1 nor AtMRP2 transported taurocholate when expressed in yeast, but taurocholate selectively inhibited DNP-GS uptake by AtMRP2 with an IC_{50} well below its CMC [71]. An important corollary of this finding, and of the facility of AtMPR2 for simultaneous, parallel transport (see above) is

that competition experiments must be interpreted with care where MRP homologues are concerned: competition does not imply that the competing compound is a bona fide substrate and conversely, absence of competition does not imply that a compound is not transported. This makes it difficult to assign significance to potentially interesting results of competition studies.

Probenecid is an anion transport inhibitor which competitively inhibits taurocholate transport in isolated vacuoles [103], and has also been reported to block vacuolar transport of anionic fluorescent dyes such as fura 2, Quin-2 and Lucifer yellow [125–128]. Lucifer yellow has been employed as a model substrate to study uptake of sulphated and sulphonated compounds into isolated rye by Klein et al. who demonstrated that transport was mediated by a MOAT-like ATPase, and was inhibited by probenecid, sulphated compounds and glucuronates, but not by glutathione conjugates [129]. Whether or not fluorescent dyes represent exogenous substrates for the taurocholate transporter, or for another vacuolar transport system, will only be confirmed by the isolation and functional expression of the relevant gene or the isolation of transport mutants. Fluorescent dyes are commonly used for probe cellular parameters such as intracellular free Ca^{2+} (fura 2, Quin-2) and pH (BCECF), and their eventual vacuolar sequestration is a significant hindrance to their use in plant cells [125,126,130]. Identification of the appropriate transporter gene(s) may lead to the construction of transgenic plants with reduced vacuolar accumulation of fluorescent probes.

Whilst the precise molecular nature of the plant taurocholate transporter remains unknown, other systems provide clues to its possible identity. Taurocholate transport has been demonstrated in secretory vesicles and a vacuole-enriched fraction of *S. cerevisiae*; the same populations of vesicles were also competent in the transport of DNP-GS, but the two substrates did not compete [131,132]. Since, as in plants, the characteristics of taurocholate transport suggested that it might be encoded by an *MRP* homologue, and yeast has several such genes, Ortiz et al. attempted to identify a taurocholate transporter genetically, by creating yeast deletion mutants lacking ABC transporters. Three candidate genes were identified by PCR, and ATP-dependent bile acid transport was abolished when one of the genes, *BAT1*, was deleted from the genome, and restored upon reintroduction of the gene [132]. BAT1 exhibited homology to *S. cerevisiae* putative ABC proteins of unknown function, to rat cMOAT, and human MRP1. Strict *BAT1* homologues have not yet been identified in plants, but the finding that rat cMOAT, which shares only 32% amino acid identity with BAT1, is able to transport bile acids [112], suggests that there may not necessarily be a great deal of sequence homology between bile acid transporters. In agreement with this suggestion, the human gene responsible for taurocholate transport, *BSEP* (bile salt export pump), has been identified by its role in disease and found to be identical to a *P-gp* homologue, *sister of P-glycoprotein* [133]. Thus, it appears that ABC transporters belonging to two different subfamilies are competent in bile acid transport.

6.3.4. Secondary products

The *Bronze-2* (*bz2*) mutation is the last genetically defined step in anthocyanin pigmentation in maize, resulting in accumulation of anthocyanins in the cytoplasm, where they are oxidised and cross-linked to form brown products. *Bz2* encodes a GST, which has been postulated to tag cyanidin-3-glucoside with glutathione, prior to sequestration by the GS-X pump [134]. In agreement with this hypothesis, the *bz2* phenotype can be mimicked by application of vanadate to wild-type protoplasts [134], and AtMRP1 and 2 have been proposed to transport a synthetic glutathionylated derivative of the anthocyanin cyanidin-3-glucoside [12,71]. A divergent, but functionally equivalent GST, *An9*, has been isolated from *Petunia*, suggesting widespread involvement of GST in anthocyanin pigmentation [135]. However, the precise role of *Bz2* is unclear, since glutathione conjugates of anthocyanins have not yet been detected in vivo, and conjugation of glutathione to cyanidin-3-glucoside by *Bz 2* has not been demonstrated. Alternative pathways for the vacuolar sequestration of anthocyanins have also been proposed: Hopp and Seitz demonstrated uptake of acylated anthocyanins into isolated carrot vacuoles [136] and a vesicular transport system for anthocyanins has been described by Grotewold et al. ([137], and refs. therein).

Other possible endogenous MRP substrates are allelochemicals such as phenolics and phytoalexins

[5,138]. The legume phytoalexin, medicarpin, when glutathionylated, has been shown to be transported into tonoplast vesicles with high efficiency [138]. Li et al. have therefore proposed a role for MRP in storage of antimicrobial compounds in healthy plant tissue [138]. Interestingly, an ABC transporter from pathogenic strains of rice blast fungus is postulated to efflux phytoalexins; however, the gene (*ABC1*) is more closely related to the yeast ABC transporter *PDR5*, than *MRP* [139].

6.4. Antiquity of GSH-mediated detoxification

The evidence for secondary metabolites as MRP substrates should not detract from a likely primary role for MRP in detoxification: whilst plants have been systematically exposed to man-made chemicals such as herbicides for the last 100 years only [111], the need to protect against cytotoxic electrophiles is ancient [95]. The occurrence of glutathione in all eukaryotes and its restriction in prokaryotes to cyanobacteria and purple bacteria suggest that it arose at the same time as oxygenic photosynthesis, as part of a mechanism to protect cells from damage by active oxygen species. Transport of oxidised glutathione (GSSG – a special form of glutathione conjugate) by GS-X pumps may thus function in defence against oxidative stress.

Many biotic and abiotic compounds are susceptible to conjugation to glutathione, since the chemical determinants of GST substrates are common and widely distributed (for discussions, see [95,140,141]), and it seems that the subsequent evolution of gene products involved in detoxification such as GSTs and GS-X pumps to perform additional functions is a consequence of the versatility of glutathione chemistry.

6.5. Tissue specificity and subcellular location

Northern analysis reveals that *AtMRP1*, *2* and *3* do not appear to have restricted distributions, being constitutively expressed in several tissues [12,71, 72,74]; expression does, however, increase in response to xenobiotic treatments [73,75,107]. Since plant MRPs accept multiple substrates, it will be informative to investigate whether a particular isoform performs different functions in different tissues or whether roles are also delineated by cell specific expression. It is already evident that certain functions of MRP are restricted to specific cell types, for example: in leaves, transport of chlorophyll catabolites is specific to the mesophyll, since the epidermis does not contain chloroplasts. However, the MRP responsible for chlorophyll catabolite transport may also be present in epidermis where it performs a different function, such as glutathione conjugate transport. Evidence for this is limited, as biochemical studies have concentrated on mesophyll vacuoles and tonoplast vesicles from whole organs; however, using leaf protoplasts, Coleman and coworkers observed that, whilst glutathione conjugates of monochlorobimane were formed in the cytosol of barley epidermal cells, they did not accumulate in the vacuole [95,142]. This may also reflect different substrate specificities of epidermal and mesophyll transporters, or may indicate that an appropriate isoform of MRP is absent from the tonoplast in epidermal cells. Experimental evidence to date strongly suggests the tonoplast as the prime location for MRP, but this does not rule out the existence of MRP isoforms in other subcellular locations.

Studies of plasma membrane GS-X transport (e.g. using inside-out plasma membrane vesicles) have not yet been reported, but recent immunological evidence points to a plasma membrane location for certain isoforms of MRP. An expression library was screened with polyclonal antibodies raised against total proteins from *Arabidopsis* plasma membrane and tonoplast, and the positive clones were used to construct two cDNA libraries enriched in genes encoding plasma membrane and tonoplast proteins respectively [143]. Sequence analysis of the plasma membrane library identified a clone 100% homologous to *AtMRP1*. Whilst this result does not necessarily indicate that AtMRP1 itself resides in the plasma membrane, it suggests that *Arabidopsis* does possess plasma membrane proteins which are antigenically related to AtMRP1. Testing of this hypothesis awaits the availability of isoform-specific antibodies, or plants transformed with epitope tagged constructs, as has been achieved for *AtPGP1* [68].

6.6. Special features of MRP

To date, cloning of plant representatives of one

ABC subclass – MRP – has accounted for several vacuolar transport activities: MRP isoforms have been shown to transport chlorophyll catabolites, and glutathione conjugates of xenobiotics, anthocyanins and phytoalexins. For other substrates, such as glucosides, involvement of an MRP is suspected, but definitive proof will require molecular cloning of the corresponding genes or analysis of mutants. The multispecific nature of MRP, and its unique ability to transport dissimilar substrates simultaneously have been significant discoveries: interestingly, unlike MDR, which can transport many substrates, but at relatively low affinity, K_m values for putative MRP substrates are all in the micromolar range [5]. Studies of how these properties are related to the specific structural features of MRP will form the basis for future investigations.

7. The search for channel regulators

The majority of ABC proteins characterised to date function as ATP-dependent pumps. However, the finding that the product of the cystic fibrosis gene is not only a chloride channel, but also a regulator of several other ion channels, opened up a new field of ABC transporter research, and challenged conventional views on the distinction between channels and pumps [92]. It was subsequently demonstrated that several other ABC proteins such as P-glycoprotein and the sulphonylurea receptor (SUR) regulate heterologous channels in addition to possessing their own intrinsic transporter activities [92], and evidence for similar phenomena has recently been sought in plants.

7.1. SUR and CFTR

The cystic fibrosis gene of animals encodes an ABC transporter with homology to MRP, but which functions as an outwardly rectifying Cl^- channel – the cystic fibrosis transmembrane conductance regulator (CFTR [144]). CFTR is regulated by cAMP-dependent phosphorylation and by ATP [144,145] and is inhibited by diphenylamine-2-carboxylic acid (DPC) [146]. CFTR itself regulates other channels in cystic fibrosis cells, including a second, distinct, outwardly rectifying Cl^- channel, [147], a Ca^{2+}-activated chloride channel [148], a sodium channel [149] and inwardly rectifying K^+ channels [150–152].

Sulphonylureas are drugs used to treat non-insulin-dependent diabetes: sulphonylurea blockade of an ATP-sensitive K^+ channel (K-ATP) in pancreatic β cells modulates insulin release. The sulphonylurea receptor (*SUR*) has been cloned and found to be an ABC transporter [8], which associates with an inwardly rectifying K^+ channel to render it sensitive to sulphonylureas and to form an ATP-sensitive K^+ channel [153]. SUR is also a receptor for K^+ channel openers, e.g. chromakalim, which can reverse the inhibitory effect of sulphonylureas [154]. Interestingly, CFTR is also blocked by the sulphonylurea glibenclamide [155,156].

Attempts have been made to clone *CFTR* and *SUR* homologues from *Arabidopsis*; however, the genes cloned by screening libraries with appropriate ESTs exhibited greater overall identity to *MRP* than *CFTR* or *SUR* and have been shown to be glutathione conjugate transporters (see above; [76]; C. Forestier, pers. comm.). These transporters may nevertheless have a second function, as MRP has been shown to alter Cl^- and K^+ currents in some mammalian cell types [157], and a recently isolated ABC channel protein, the epithelial basolateral cAMP-regulated Cl^- channel conductance regulator (EBCR), exhibits greater homology to MRP isoforms than to CFTR [158].

Electrophysiological approaches to study plant ABC transporter/channel relations have proved more informative: guard cells possess a number of well-characterised channel activities implicated in stomatal movements [159], some of which may represent ABC transporters: for example, anion channels have been proposed to be *CFTR* homologues [160,161]. Thus they comprise an excellent model system to study ABC transporter/channel interactions in plants [162].

Forestier and coworkers have exploited the specific pharmacological profile of K-ATP and CFTR to probe possible analogous systems in stomatal guard cells. In one study, the effect of sulphonylureas and K^+ channel openers on guard cell K^+ channels was investigated [162]. The sulphonylureas, tolbutamide and glibenclamide induced stomatal opening in epidermal strip bioassays, and in whole cell patch clamp experiments, glibenclamide was found to inhibit the

outward K^+ current, but did not affect the inward K^+ current. Conversely, K^+ channel openers such as chromakalim triggered stomatal closure under light and prevented stomatal opening, suggesting that K^+ permeability through the outward K^+ channel could be increased. Sulphonylureas and K^+ channel openers competed in the epidermal strip bioassays, and taken together, the results suggest the presence of a SUR-like protein in guard cell plasma membrane, which is involved in the regulation of the outward K^+ channel during stomatal movements. The experimental conditions employed did not permit unequivocal identification of an ATP-dependent K^+ channel, since ATP was present in pipette solutions, and in a previous study, the guard cell outward K^+ channel was not found to be ATP dependent [163]. Further investigation of the presence of K-ATP in guard cells awaits the molecular characterisation of the K^+ outward rectifier from guard cells. A potassium outward rectifier (*KCO1*) has been cloned from *Arabidopsis*, where it is expressed in leaves [164]. This gene belongs not to the ABC superfamily, but to a new group of 'two-pore' K^+ channels [164] – it would be interesting to test whether KCO1 interacts with ABC transporters to yield an ATP-dependent K^+ channel. A Shaker-type outward rectifying K^+ channel gene, *SKOR*, has also been isolated from plants, but its expression is specific to the root stele [165]. When making comparisons between ABC/K^+ channel interactions in plant and animal systems, it should be noted that the K^+ channel induced by SUR in mammalian cells is an *inward* rectifier [153] and belongs to a different gene family to *KCO1* [164]. A potassium inward rectifier has been shown to play a critical role in stomatal opening [166], and Shaker-type *Arabidopsis* and potato genes encoding K^+ inward rectifiers are indeed expressed in guard cells [167,168]. Although the K^+ inward rectifier was not affected by sulphonylureas in the experiments of Leonhardt et al. [162], the question of whether this channel is modulated by ABC transporters remains open.

In guard cells, slow anion channels are activated by Ca^{2+}, and are thought to mediate the prolonged anion efflux necessary for stomatal closure [169,170]. Again, using whole-cell patch clamp experiments and epidermal strip bioassays, Leonhardt et al. studied the effect of ABC/channel modulators on the slow anion channel [171]. CFTR blockers glibenclamide and DPC were found to inhibit the slow anion current, and triggered stomatal opening in darkness. The potassium channel opener, chromakalim, suppressed glibenclamide-induced stomatal opening, and recovered the glibenclamide-inhibited slow anion current. Glibenclamide prevented stomatal closure triggered by Ca^{2+} and ABA, and, accordingly, ABA partially relieved glibenclamide inhibition of the slow anion current, suggesting interactions with Ca^{2+} and ABA signalling cascades. Overall, the results of the two guard cell studies confirm that the guard cell slow anion channel is similar to CFTR, and suggest that it mediates sulphonylurea block of the outward K^+ channel. This interaction may allow coordinated efflux of K^+ and anions during stomatal closure, and is analogous to the interaction between mammalian CFTR and Na^+ channels [92].

7.2. P-gp channel activity

In addition to its role as an ATP-dependent drug efflux pump, mammalian P-glycoprotein has been shown to be associated with a volume-activated chloride channel [172,173]. P-gp alters the sensitivity of channel activation to osmotic gradients and can impose protein kinase C dependency on channel activation. Three lines of evidence confirm that channel regulator and drug transport functions are distinct activities: requirements for ATP hydrolysis (drug transport requires ATP hydrolysis, whilst channel function can be supported by non-hydrolysable analogues [173]), pharmacology [174] and differential regulation by phosphorylation: protein kinase C-mediated phosphorylation regulates channel activity, but not drug transport [175,176]. No channel regulating activity has yet been ascribed to P-gp plant homologues.

7.3. Prospects for plants

Given the presence of P-gp and MRP transporters in plants, it is possible that some of these ABC proteins interact with ion channels, as has been demonstrated in mammalian systems. Despite a growing body of pharmacological evidence for ABC/channel interactions in guard cells [162,171], no *cloned* plant ABC transporter has yet been shown to exhibit chan-

nel or channel regulator activity. Several groups are currently investigating this possibility by expressing ABC transporters in heterologous expression systems such as *Xenopus* oocytes. However, since effects of ABC transporters on channel activity can be quite subtle, e.g. increased sensitivity to ATP, as opposed to activation/deactivation, channel-transporter interactions may prove difficult to detect in heterologous systems, without clues from other experiments to the identity of the channel activity in question. Also, not all ABC proteins are expected to have dual functions: in view of the size and diversity of the superfamily, this would clearly be deleterious for the cell [1]. Thus, the extent of this phenomenon remains to be determined, both in mammalian and plant systems, and an unequivocal estimation will be hard to achieve. Application of techniques such as two hybrid systems, 'green Westerns', and coexpression of libraries with cloned genes in heterologous systems has already helped to identify proteins which interact with various membrane transporters – development and use of these techniques to study ABC transporters may lead to progress in the field of channel regulation in the future.

8. PDR5 subfamily

Whilst several plant ABC transporters have been cloned by homology to genes from yeast and humans, or following their identification in genome sequencing projects, differential screening studies of two developmental processes have led to the identification of a new class of plant ABC transporter homologues. In the aquatic plant, *Spirodela polyrrhiza*, formation of dormant buds, termed turions, is induced by ABA treatment and low temperature, and the effect of ABA is antagonised by cytokinins [177,178]. In a screen for transcripts induced by ABA, Smart and Fleming isolated a homologue of the yeast drug resistance gene: *PDR5* [78]. PDR5 is a 'mirror image' four-domain ABC protein, which mediates plasma membrane efflux of numerous unrelated toxins in yeast ([23]; see above). The plant homologue, *TUR2*, was expressed at very low levels in untreated tissue, but expression appeared to be correlated to factors involved in the control of turion formation, since transcripts were induced by ABA and repressed by kinetin. However, in situ analysis revealed that *TUR2* mRNA accumulated in all parts of the plant, not just in those involved in turion formation, and could also be induced by stress treatments such as low temperature and high salt. The authors concluded that the accumulation of *TUR2* transcripts was associated with the decrease in growth which accompanies turion formation, rather than a causal event.

Similarly, a study of changes in gene expression during the onset of somatic embryogenesis led to the identification a *SNQ2* homologue in alfalfa [79]. SNQ2 is a plasma membrane-bound ABC transporter, which is closely related to PDR5, and also mediates drug resistance in yeast [24,25]. The alfalfa *SNQ2* homologue was expressed 10 days after induction of embryogenesis by 2,4-D and wounding; it would therefore be interesting to investigate further the response of this gene to stress and hormones, in the light of the expression patterns of *TUR2* [78].

At present, the role of the PDR5 subfamily in plants is not known. The regulation of *TUR2* and alfalfa *SNQ2* by plant hormones is an interesting feature, since yeast SNQ2 has been shown to transport steroid hormones in addition to cytotoxins [179]. However, it is premature to suggest similar roles for the plant transporters. Homologues of *TUR2* have been identified in the *Arabidopsis* and rice EST databases, indicating their widespread presence in higher plants, and permitting detailed molecular analysis, which may help elucidate their precise roles in physiology ([78]; C. Smart, pers. comm.).

9. Organelle ABC transporters

9.1. Chloroplast

Genome sequencing projects have led to the discovery of several putative organellar ABC transporters; indeed, the first plant ABC gene to be identified was a 1.1 kb reading frame, *mbpX*, in the *Marchantia* chloroplast genome [86,87]. *MbpX* encodes a bacterial permease-like membrane-spanning subunit, and a candidate for another component of a putative complex containing the *mbpX* product is encoded in a second, linked reading frame, *mbpY* [88]. Reading frames encoding ATP binding subunits have also

been identified in the plastid genomes of red algae [84,85] and diatoms [27]. Not all organellar genes are transcribed, but immunoprecipitation studies indicated that the diatom protein, YCF16, is synthesised in plastids and located in the stroma [27]. Analysis of sequenced chloroplast genome of higher plants has not revealed the presence of *mbpX* or *ycf16* homologues, suggesting either that the proteins encoded by these genes are not required in higher plants, or that they have been transferred to the nuclear genome during the course of evolution [27]. Such 'migration' of sequences from organellar genomes to the nucleus is well documented [180], and thus the latter explanation is plausible.

9.2. Mitochondria

ABC transporter subunit genes implicated in the biogenesis of cytochrome *c* have recently been identified in higher plant mitochondrial genomes. Cytochromes *c* are located outside the cytoplasmic membrane of bacteria, in the intermembrane space of mitochondria, and in the lumen in chloroplasts and their assembly therefore requires transmembrane transport of haem [181]. The photosynthetic bacterium *Rhodobacter capsulatus* has been employed as a model organism to study cytochrome *c* biosynthesis, and *helABC* genes encoding components of an ABC transporter necessary for haem export and ligation have been characterised in detail [182]. As *Rhodobacter* is phylogenetically related to endosymbiont ancestors, it is perhaps not surprising that orthologues of the two transmembrane subunits encoded by *helB* and *C* have been identified in the mitochondrial genome of *Marchantia* [80]. Orthologues are also present in several higher plant species (see Table 1; [28,29,81–83,183]). However, the gene encoding the ATP binding subunit, *helA*, has not been identified in any of the mitochondrial genomes sequenced to date, and it has been proposed that this and other genes involved in cytochrome biogenesis have been transferred to the nucleus [181].

9.3. Nuclear-encoded ABC transporters

Despite the paucity of organellar ABC transporter genes, the presence of and requirement for further ABC proteins in organelles can be postulated. Firstly, preliminary experimental evidence suggests that additional ABC transporters exist, for example, antibodies raised to conserved regions of P-glycoprotein react positively in immunoblots of chloroplast outer envelope membranes [184]. Furthermore, chloroplast MRP homologues may be implicated in chlorophyll degradation. As discussed above, it has been shown that AtMRP2 and 3 are capable of mediating transport of the chlorophyll metabolite *Bn*-NCC-1 into the vacuole when expressed in transgenic yeast [71,74] and biochemical studies have identified an MRP-like *Bn*-NCC-1 vacuolar transport activity in barley [117]. Since chlorophyll breakdown products must somehow exit the plastid before this can take place, a second ABC transporter may also exist in the chloroplast envelope. In agreement with this, chlorophyll catabolites have been shown to be released from intact barley gerontoplasts into the medium in an ATP-dependent fashion [185].

10. ABC transporters in plant-microbe interactions

In addition to endogenous transporters, a number of ABC proteins are relevant to plant biology by virtue of their role in symbiotic and pathogenic relationships. *Rhizobium* and *Agrobacterium* are closely related bacteria whose associations with higher plants have become important paradigms for studying plant-microbe interactions: agrobacteria are tumour-inducing pathogens of dicotyledonous plants, and rhizobia are involved in symbiotic nitrogen fixation in legume root nodules. Such intimate associations require the exchange of signals and nutrients between plant and microorganism, and by definition must involve numerous transport processes. Biochemical and genetic studies have demonstrated a number of ABC transporters in the Rhizobiaceae family which operate at different stages of the association.

10.1. Agrobacterium

During *Agrobacterium*-mediated gall formation, a piece of bacterial DNA – the T-DNA – is transferred to the plant cell, where it becomes stably integrated into the host genome. Components encoded by the *vir* regulon are responsible for this process. *Vir* genes

are induced in response to chemical signals of the plant wound site, for example, monosaccharides are bound by a periplasmic binding protein, ChvE, which then interacts with the membrane-spanning VirA molecule of the VirA-VirG sensor-regulator pair to activate transcription of the *vir* regulon. As part of this process, ChvE also mediates chemotaxis towards sugars, through interaction with an unidentified receptor. Additionally, analysis of the region downstream from the *ChvE* gene indicates that it is also part of an ABC sugar transport operon, thus one periplasmic binding protein serves three related functions in establishment of the association: signalling, chemotaxis and sugar uptake [33].

Overlap between nutrition and signalling also occurs later in tumour development: the integrated T-DNA directs the synthesis of opines – amino acid and sugar derivatives which are produced by the plant and taken up and catabolised by the infecting bacteria. ABC genes encoding periplasmic transport systems for octopine and nopaline have been cloned, and found to be homologous to the histidine permease of *S. typhimurium* [186,187]. In addition to acting as nutritional sources, a subclass of opines function as signal molecules, and have a specific ABC uptake system [188].

Interestingly, the induction of tumours by *Agrobacterium tumefaciens* and the production of nitrogen-fixing nodules by *Rhizobium meliloti* require a related set of genes. Several linked chromosomal genes (*chvA*, *chvB*, *exoC*), are required for attachment of *A. tumefaciens* to plant cells and have homologous, functionally interchangeable counterparts (*ndvA*, *ndvB*, *exoC*) in *R. meliloti* [189]. Mutagenic analysis has implicated *chvB*/*ndvB* in the synthesis of a low molecular weight, cyclic β-1,2-glucan required for attachment, but mutants of *chvA* and *ndvA* also fail to produce extracellular polysaccharide. The *chvA*/*ndvA* loci have been sequenced and found to encode a polypeptide homologous to the haemolysin export protein of *E. coli*, suggesting a role in polysaccharide export, and this has been confirmed experimentally [190,191].

10.2. Rhizobium

Further transport parallels between tumour and nodule formation also exist: the capacity to utilise specific carbon sources and the ability to respond to signals are also mediated by ABC transporters in rhizobia. One of the first steps in plant-*Rhizobium* signal exchange is the induction of plasmid-borne *nod* genes by plant-exuded flavonoids or betaines – a phenomenon which requires as yet unidentified transporters in both plant and bacterium. In light of the ability of flavonoids to act as substrates for mammalian and yeast ABC transporters, it is tempting to speculate that an ABC transporter might be responsible for export of flavonoids from the root.

Nod genes are involved in the synthesis of nod factors, which trigger the nodulation process. Most nod factors are lipochitooligosaccharides, but nodO, which is specific to *R. leguminosarum* bv. *viciae*, is a protein. Downie and coworkers have demonstrated that nodO is transported from the cell by an ABC system: nodO could be exported by a strain of *E. coli* carrying the *nodO* gene, the outer membrane protein gene, *tolc*, plus either the haemolysin transporter genes *hlyBD*, or the protease secretion genes, *prtDEF* from *Erwinia chrysanthami* [192]. Although *nodO* homologues are not found in other rhizobia, several strains were found to have the ability to export nodO, which is consistent with the finding that the protein transporter genes are unlinked to the *nod* genes, and may represent a general protein transport apparatus [192]. Interestingly, three genes encoding a ABC transporter with homology to *prtDE* were recently isolated from the genome of *R. leguminosarum* [193].

Other *Rhizobium* loci related to nodulation but not involved in nod factor production have been described; some of these act to ensure the survival of the bacteria in the rhizosphere, such as the *R. meliloti* locus *ndvF* which encodes an ABC phosphate uptake system, and appears to play a purely nutritional role in nodulation [194]. Others may be involved in signalling processes between bacteria and plants to establish colonisation of the root, for example: in a search for *Rhizobium tropici* genes inducible by host exudates, Rosenblueth et al. isolated an ABC transport complex with homology to ribose transport proteins, thought to mediate host-specific exudate uptake [195].

10.3. Fungal pathogens

Whilst the role of fungal ABC transporters in drug resistance phenomena is well established [55], their involvement in pathogenic interactions with plants is only beginning to emerge. An ABC transporter (*ABC1*) was recently identified in an insertional mutagenesis screen for pathogenicity mutants of the rice blast fungus, *Magnaporthe grisea* [138]. ABC1 is 47% identical to the well-characterised yeast ABC transporter, PDR5, which is known to operate as a drug efflux pump [55]. Both *ABC1* and *PDR5* transcript levels are dramatically elevated in response to metabolic poisons, and up-regulation of ABC1 was required for pathogenicity. However, unlike *PDR5*, deletions in *ABC1* did not result in sensitivity to metabolic poisons and antifungals, implying a specialised pathogenic function, rather than a general role in drug resistance. Homology to PDR5 suggests that ABC1 may act to export a fungal toxin during pathogenesis, but this seems unlikely since there is no evidence for toxin production in rice blast disease, and growth of abc1 mutants was arrested following penetration of the plant, a phenomenon which is not observed in Tox minus strains of other fungal pathogens. The authors therefore proposed that ABC1 acts as an efflux pump to remove antimicrobial compounds, such as phytoalexins present in rice, and may therefore represent a common phenomenon in plant pathogenesis.

10.4. General relevance

Given the examples described above, it seems axiomatic that the transport complement of a microorganism can contribute to its symbiotic and saprophytic competence. These observations can be extended to other systems, for example an ABC transporter has been shown to play a defensive role in protecting lactobacilli from toxins produced by hops [196] and an ABC transporter has been implicated in the *Pseudomonas*-oilseed rape mutualism [197].

11. Conclusions and future perspectives

In the last decade, the existence of several classes of plant ABC transporters has been established. Whilst much has been learned already, many questions remain: What are the prospects for the identification, cloning and characterisation of further transporters? What are the roles of those transporters already isolated? How do the properties of these proteins reflect their structure? How are the ABC transporters regulated? To what extent is the complement of ABC transporters specific to plants? These questions and others will help form the background of plant ABC transporter research in the future.

11.1. Cloning and characterisation of plant ABC transporters

Plant ABC transporters have been identified and cloned by a variety of means: many have been identified as a result of genome sequencing and EST projects, a number have been isolated by PCR; other, novel clones have emerged in differential screens. Many plant ABC transporters will continue to be identified as plant genome sequencing projects approach completion. Characterisation of these transporters, for which only sequence information is available, presents a challenge for biologists. The increasing availability of tagged mutants is a tool with which to address this problem, but identifying or isolating clones with reference to their function circumvents problems with characterisation. Subtractive cloning has the advantage that it may afford clues to the identity of the transporter, over and above its homology to other proteins. Also, yeast complementation has been a popular and successful method for cloning many plant transporter cDNAs in recent years [198], and the superb variety of available yeast ABC transporter mutants (e.g. [57]) makes functional cloning appear appealing for plant members of this superfamily. However, the large size (120–170 kDa; 3–6 kb) of four-domain ABC transporters such as MDR and MRP may prove prohibitive in functional cloning experiments. In an attempt to clone an *Arabidopsis* STE 6 homologue by complementation, Covic and Lew isolated a serine/threonine protein kinase [199]. While this result is interesting and has yielded a novel non-transport gene, it highlights the difficulty of obtaining transporter clones. The use of size-selected libraries may facilitate functional cloning, but isolating full-length

transporters from a directional library may prove problematic due to leaky, toxic expression when the library is propagated in *E. coli*. Whilst yeast may not be the first choice for cloning, it nevertheless remains a useful host for heterologous expression of cloned ABC transporter genes, as has been the case for MRP homologues. For other transporter subclasses, judicious use of transcription factor mutants to remove several endogenous ABC transporters will facilitate analysis. Also, yeast ABC transporter genes are also a potential source of useful promoters for heterologous expression: the PDR5 promoter having proved particularly strong [65].

11.2. Extent of the ABC superfamily in plants; lessons from other organisms

Given the size of ABC superfamilies in microbes and humans, and the range of suspected roles for ABC transporters, the identification of further plant ABC genes is highly likely. However, it is risky to base speculations concerning the size of a gene family on comparisons with other organisms: within bacteria alone, the size of the ABC family varies 5-fold [2]. It is also difficult to speculate accurately on the possible roles of the ABC transporters. Nevertheless, comparative genomics can be extremely instructive when interpreted with caution, as has been shown for comparisons of the yeast and *Caenorhabditis* genomes [67]: it is expected that the ABC transporter complement of an organism will include 'housekeeping' proteins necessary for cellular maintenance – these will have orthologues throughout eukaryotes, but it will also include proteins which have specialised roles in the host organism. This is the case for other gene families, for example: the complete *Saccharomyces* genome sequence reveals only three genes encoding cytochromes *P*-450, whereas 275 have been identified to date in *Arabidopsis* [200,201]; such discrepancies reflect the different metabolic activities of the two groups of organisms, in this case, the extensive ability of plants to synthesise secondary metabolites. The ABC transporter complement of plants may similarly play a special role in secondary metabolism.

In other cases, housekeeping functions common to eukaryotes may be achieved by different means in different organisms, depending on their lifestyles: ABC transporters which mediate detoxification are a good example of this. Since yeast is unicellular, it deals with toxic insults largely by exclusion, effluxing compounds into the extracellular medium, and therefore has a complex network of inducible plasma membrane ABC transporters. Multicellular animals can also effectively exclude toxins from their cells, since these can be excreted from the body. However, toxins are metabolised prior to transport and are effluxed by rather different ABC proteins. Plants, in contrast, have evolved to accommodate their sessile habit: they contain a similar complement of detoxification enzymes to that found in mammalian liver [94], but rather than being excreted, toxins are metabolised and sequestered intracellularly. Such differences cannot be deduced from sequence databases alone.

11.3. A final word

To a large extent, research on plant ABC transporters has been directed by analogy to mammalian and microbial systems. Whilst much has been learned from comparative studies, there are aspects of biology which are specific to plants, and cannot be addressed by comparison with other organisms. It may be that some of the most interesting ABC transporters are those which are specific to plants. It may also be the case that novel information relevant to all organisms emerges from plant ABC transport research.

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References

[1] C.F. Higgins, Annu. Rev. Cell Biol. 8 (1992) 67–113.
[2] A. Decottignies, A. Goffeau, Nat. Genet. 15 (1997) 137–145.
[3] K.J. Linton, C.F. Higgins, Mol. Microbiol. 28 (1998) 5–13.
[4] M. Dean, R. Allikmets, Curr. Opin. Genes Dev. 5 (1995) 779–785.
[5] P.A. Rea, Z.-S. Li, Y.-P. Lu, Y.M. Drozdowicz, E. Martinoia, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 727–760.
[6] G.F.-L. Ames, Annu. Rev. Biochem. 55 (1986) 397–425.
[7] J.M. Rommens, M.C. Iannuzzi, B. Kerem, M.L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J.L. Cole, D. Kennedy, N. Hidaka, M. Zsiga, M. Buchwald, J.R. Riordan, L.C. Tsui, F.S. Collins, Science 245 (1989) 1059–1065.
[8] L. Aguilar-Bryan, C.G. Nichols, S.W. Wechsler, J.P. Clement, A.E. Boyd, G. Gonzalez, H. Herrera-Sosa, K. Nguy, J. Bryan, D.A. Nelson, Science 268 (1995) 423–426.
[9] R. Prasad, S.K. Murthy, R. Prasad, V. Gupta, S. Lata, Curr. Sci. 71 (1996) 205–213.
[10] S.P.C. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M.V. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[11] C.J. Chen, J.E. Chin, K. Ueda, D.P. Clark, I. Pastan, M.M. Gottesman, I.B. Robinson, Cell 47 (1986) 381–389.
[12] Y.-P. Lu, Z.-S. Li, P.A. Rea, Proc. Natl. Acad. Sci. USA 92 (1997) 8243–8248.
[13] R.W. Dudler, C. Hertig, J. Biol. Chem. 267 (1992) 5882–5888.
[14] J.E. Walker, M. Saraste, M.J. Runswick, N.J. Gay, EMBO J. 1 (1982) 945–951.
[15] C.F. Higgins, I.D. Hiles, G.P.C. Salmond, D.R. Gill, J.A. Downie, I.J. Evans, I.B. Holland, L. Gray, S.D. Buckel, A.W. Bell, M.A. Hermondson, Nature 323 (1986) 448–450.
[16] R. Dudler, M. Sidler, Methods Enzymol. 292 (1998) 162–173.
[17] S.C. Hyde, P. Emsley, M.J. Hartshorn, M.M. Mimmack, U. Gileadi, S.R. Pearce, M.P. Gallagher, D.R. Gill, R.E. Hubbard, C.F. Higgins, Nature 346 (1990) 362–365.
[18] K. Kuchler, R.E. Sterne, J. Thorner, EMBO J. 8 (1989) 3973–3984.
[19] J. Leighton, G. Schatz, EMBO J. 14 (1995) 188–195.
[20] P. Parham, Nature 351 (1991) 271–272.
[21] C.R. Vazquez de Aldana, M.J. Marton, A.G. Hinnebusch, EMBO J. 14 (1995) 3184–3199.
[22] G. Leppert, R. McDervitt, S.C. Falco, T.K. Van Dyk, M.B. Ficke, J. Golin, Genetics 125 (1990) 13–20.
[23] E. Balzi, M. Wang, S. Leterme, L. Van Dyck, A. Goffeau, J. Biol. Chem. 269 (1994) 2206–2214.
[24] J. Servos, E. Haase, M. Brendel, Mol. Gen. Genet. 236 (1993) 214–218.
[25] A. Decottignies, L. Lambert, P. Catty, H. Degand, E.A. Epping, W.S. Moye-Rowley, E. Balzi, A. Goffeau, J. Biol. Chem. 270 (1995) 18150–18157.
[26] G.D. Ewart, D. Cannell, B.C. Cox, A.J. Howells, J. Biol. Chem. 269 (1994) 10370–10377.
[27] K. Wittpoth, P.G. Kroth-Pancic, H. Stotmann, Plant Sci. 114 (1996) 171–179.
[28] W. Schuster, Plant Mol. Biol. 25 (1994) 33–42.
[29] G. Bonnard, J.M. Grienenberger, Mol. Gen. Genet. 246 (1995) 91–99.
[30] T. Salehzada, M. Sihol, A.M. Steff, B. Lebleu, C. Bisbal, J. Biol. Chem. 268 (1993) 7733–7740.
[31] S.H. Cheng, D.P. Rich, J. Marshall, R.J. Gregory, M.J. Welsh, A.E. Smith, Cell 66 (1991) 1027–1036.
[32] D.P. Rich, R.J. Gregory, M.P. Andeson, P. Manavalan, A.E. Smith et al., Science 253 (1991) 205–207.
[33] J.M. Kemner, X. Liang, E.W. Nester, J. Bacteriol. 179 (1997) 2452–2458.
[34] T.W. Loo, D.M. Clarke, J. Biol. Chem. 270 (1995) 843–848.
[35] T.W. Loo, D.M. Clarke, J. Biol. Chem. 271 (1996) 15414–15419. Y.-P. Lu, O.K. Vatamaniuk, P.A. Rea, Exp. Biol. Online 3 (1998).
[36] C. Kast, V. Canfield, R. Levenson, P. Gros, Biochemistry 34 (1995) 4402–4411.
[37] G.E. Tusnády, E. Bakos, A. Varadi, B. Sarkadi, FEBS Lett. 402 (1997) 1–3.
[38] E. Bakos, T. Hegedüs, Z. Holló, E. Welker, G.A. Tusnády, G.J.R. Zaman, M.J. Flens, A. Váradi, B. Sarkadi, J. Biol. Chem. 271 (1996) 12322–12326.
[39] D.R. Hipfner, K.C. Almquist, E.M. Leslie, J.H. Gerlach, C.E. Grant, R.G. Deeley, S.P.C. Cole, J. Biol. Chem. 272 (1997) 23623–23630.
[40] C. Kast, P. Gros, J. Biol. Chem. 272 (1997) 26479–26487.
[41] C. Kast, P. Gros, Biochemistry 37 (1998) 2305–2313.
[42] M.F. Rosenberg, R. Callaghan, R.C. Ford, C.F. Higgins, J. Biol. Chem. 272 (1997) 10685–10694.
[43] J.P. Annereau, U. Wulbrand, A. Vankeerberghen, H. Cuppens, F. Bontems, B. Tummler, J.J. Cassiman, V. Stoven, FEBS Lett. 407 (1997) 303–308.
[44] M.A. Bianchet, Y.H. Ko, L.M. Amzel, P.L. Pedersen, J. Bioenerg. Biomembr. 29 (1997) 503–524.
[45] F.J. Hoedermaeker, A.R. Davidson, D.R. Rose, Proteins 30 (1998) 275–286.
[46] L.-W. Hung, I.X. Wang, K. Nikaido, P.-Q. Liu, G. Ferro-Luzzi Ames, S.-H. Kim, Nature 396 (1998) 703–707.
[47] C.F. Higgins, M.M. Gottesman, Trends Biochem. Sci. 17 (1992) 18–21.
[48] J.J.M. Smit, A.H. Schinkel, R.P.J. Oude Elferink, A.K. Groen, E. Wagenaar, L. van Deemter, C.A.A.M. Mol, R. Ottenhofer, N.M.T. van der Lugt, M.A. van Roon, M.A. van der Walk, G.J.A. Offerhaus, A.J.M. Berns, P. Borst, Cell 75 (1993) 451–462.
[49] S. Ruetz, P. Gros, Cell 77 (1994) 1071–1081.
[50] A. van Helvoort, A.J. Smith, H. Sprong, I. Fritzsche, A.H. Schinkel, P. Borst, G. van Meer, Cell 87 (1996) 507–517.
[51] I. Leier, G. Jedlitschky, U. Buchholz, S.P.C. Cole, R.G. Deeley, D. Keppler, J. Biol. Chem. 269 (1994) 27807–27810.
[52] M. Müller, C. Meijer, G.J.R. Zaman, P. Borst, R.J. Scheper, N.H. Mulder, E.G.E. de Vries, P.L.M. Jansen, Proc. Natl. Acad. Sci. USA 91 (1994) 13033–13037.
[53] G.J. R Zaman, M.J. Flens, M.R. van Leusden, M. de Haas,

H.S. Mülder, J. Lankelma, H.M. Pinedo, R.J. Scheper, F. Baas, H.J. Broxterman, P. Borst, Proc. Natl. Acad. Sci. USA 91 (1994) 8822.
[54] S. Paul, L.M. Breuninger, K.D. Tew, H. Shen, G.D. Kruh, Proc. Natl. Acad. Sci. USA 93 (1996) 6929–6934.
[55] E. Balzi, A. Goffeau, Biochim. Biophys. Acta 1187 (1994) 152–162.
[56] F. Wendler, H. Bergler, K. Prutej, H. Jungwirth, G. Zisser, K. Kuchler, G. Hogenauer, J. Biol. Chem. 272 (1997) 27091–27098.
[57] M. Kolaczkowski, A. Kolaczkowska, J. Luczynski, S. Witek, A. Goffeau, Microb. Drug Resist. 4 (1998) 143–158. A. Krall, S.P. Bohen, K.R. Yamamoto, Proc. Natl. Acad. Sci. USA 92 (1995) 4701–4705.
[58] J.A. Wemmie, M.S. Szczypka, D.J. Thiele, W.S. Moye-Rowley, J. Biol. Chem. 269 (1994) 32592–323597.
[59] M.S. Szczypka, J.A. Wemmie, W.S. Moye-Rowley, D.J. Thiele, J. Biol. Chem. 269 (1994) 22853–22857.
[60] Z.-S. Li, M. Szczypka, Y.-P. Lu, D.J. Thiele, P.A. Rea, J. Biol. Chem. 271 (1996) 6509–6517.
[61] Z.-S. Li, Y.-P. Lu, R.-G. Zhen, M. Szczypka, D.J. Thiele, P.A. Rea, Proc. Natl. Acad. Sci. USA 94 (1997) 42–47.
[62] D.J. Katzmann, T.C. Hallstrom, M. Voet, W. Wysock, J. Golin, G. Volcaert, W.S. Moye-Rowley, Mol. Cell. Biol. 15 (1995) 6875–6883.
[63] D. Hirata, K. Yano, K. Miyahara, T. Miyakawa, Curr. Genet. 26 (1994) 285–294.
[64] P.H. Bissinger, K. Kuchler, J. Biol. Chem. 269 (1994) 4180–4186.
[65] A. Decottignies, A.M. Grant, J.W. Nichols, H. de Wet, D.B. McIntosh, A. Goffeau, J. Biol. Chem. 273 (1998) 12612–12622.
[66] H. Wolfger, Y. Mahé, A. Parle-McDermott, A. Delahodde, K. Kuchler, FEBS Lett. 418 (1997) 269–274.
[67] S.A. Chervitz, L. Aravind, G. Sherlock, C.A. Ball, E.V. Koonin, S.S. Dwight, M.A. Harris, K. Dolinski, S. Mohr, T. Smith, S. Weng, J.M. Cherry, D. Botstein, Science 282 (1998) 2022–2028.
[68] M. Sidler, P. Hassa, S. Hasan, C. Ringli, R. Dudler, Plant Cell 10 (1998) 1623–1636.
[69] W. Wang, D. Takezawa, B.W. Poovaiah, Plant Mol. Biol. 31 (1996) 683–687.
[70] T.G.E. Davies, F.L. Theodoulou, D.L. Hallahan, B.G. Forde, Gene 199 (1997) 195–202.
[71] Y.-P. Lu, Z.-S. Li, Y.M. Drozdowicz, S. Hörtensteiner, E. Martinoia, P.A. Rea, Plant Cell 10 (1998) 267–282.
[72] E: Marin, N. Leonhardt, A. Vavasseur, C. Forestier, Biochim. Biophys. Acta 1369 (1998) 7–13.
[73] R. Tommasini, E. Vogt, J. Schmid, M. Fromenteau, N. Amrhein, E. Martinoia, FEBS Lett. 411 (1997) 206–210.
[74] R. Tommasini, E. Vogt, M. Fromenteau, S. Hörtensteiner, P. Matile, N. Amrhein, E. Martinoia, Plant J. 13 (1998) 773–780.
[75] R. Sánchez-Fernández, W. Ardiles-Díaz, M. Van Montagu, D. Inzé, M.J. May, Mol. Gen. Genet. 258 (1998) 655–662.
[76] N. Weigmann, M. Ansorge, E. Martinoia, B. Müller-Röber, Exp. Biol. Online 3 (1997) 110.
[77] F.L. Theodoulou, I.M. Clark, K.E. Pallett, D.L. Hallahan, Exp. Biol. Online 3 (1997) 77.
[78] C.C. Smart, A.J. Fleming, J. Biol. Chem. 271 (1996) 19351–19357.
[79] M.R. Fowler, L.M. Ong, E. Russinova, A.I. Atanassov, N.W. Scott, A. Slater, M.C. Elliott, J. Exp. Bot. 49 (1998) 249–253.
[80] K. Oda, K. Yamato, E. Ohta, Y. Nakaamura, M. Takemura, N. Nozato, K. Akashi, T. Kanegae, Y. Ogura, T. Kohchi, K. Ohyama, J. Mol. Biol. 223 (1992) 1–7.
[81] Y. Shikanai, S. Nakata, K. Harada, K. Watanabe, Plant Cell Physiol. 37 (1996) 692–696.
[82] M. Nakazono, Y. Ito, N. Tsutsumi, A. Hirai, Curr. Genet. 29 (1996) 412–416.
[83] W. Jekabsons, W. Schuster, Mol. Gen. Genet. 246 (1995) 166–173.
[84] M. Kostrzewa, K. Zetsche, J. Mol. Biol. 227 (1992) 961–970.
[85] M. Kostrzewa, K. Zetsche, Plant Mol. Biol. 23 (1993) 67–76.
[86] K. Ohyama, H. Fukuzawa, T. Kohchi, H. Shiria, T. Sano, S. Sano, K. Umesono, Y. Shiki, M. Takeuchi, Z. Chang, S.-I. Aota, H. Inokuchi, H. Ozeki, Nature 322 (1986) 572–574.
[87] K. Umesono, H. Inokuchi, Y. Shiki, M. Takeuchi, Z. Chang, H. Fukuzawa, T. Kohchi, H. Shirai, K. Ohyama, H. Ozeki, J. Mol. Biol. 203 (1988) 299–331.
[88] T. Kohchi, H. Shirai, H. Fukuzawa, T. Sano, T. Komano, K. Umesono, H. Inokuchi, H. Ozeki, K. Ohyama, J. Mol. Biol. 203 (1988) 353–372.
[89] S.R. Moss, G.W. Cussans, in: M. Ford, D. Holloman, B. Khambay, R. Sawicki (Eds.), Biological and Chemical Approaches to Combating Resistances to Xenobiotics, Society for Chemical Industry, London, 1990, pp. 200–213.
[90] E. Martinoia, E. Grill, R. Tommasini, K. Kreuz, N. Amrhein, Nature 364 (1993) 247–249.
[91] P.A. Rea, J. Exp. Bot. 50 (1999) 895–913.
[92] C.F. Higgins, Cell 82 (1995) 693–696.
[93] D. Bouchez, H. Höfte, Plant Physiol. 118 (1998) 725–732.
[94] K. Kreuz, R. Tommasini, E. Martinoia, Plant Physiol. 111 (1996) 349–353.
[95] J.O.D. Coleman, M.M.A. Blake-Kalff, T.G.E. Davies, Trends Plant Sci. 2 (1997) 144–151.
[96] K. Kobayashi, Y. Sogame, H. Hara, K.J. Hazashi, J. Biol. Chem. 265 (1990) 7737–7741.
[97] T. Kitamura, P. Jansen, C. Hardenbrook, Y. Kamimoto, Z. Gatmaitan, I.M. Arias, Proc. Natl. Acad. Sci. USA 87 (1990) 3557–3561.
[98] T.P. Akerboom, V. Narayanaswami, M. Kunst, H. Sies, J. Biol. Chem. 266 (1991) 13147–13152.
[99] G.L. Lamoureux, R.M. Shimabukuro, D.S. Frear, in: J.C. Caseley, G.W. Cussan, R.K. Atkin (Eds.), Herbicide Resistance in Weeds and Crops, Butterworth-Heinemann, Oxford, 1991, pp. 227–261.

[100] R. Tommasini, E. Martinoia, E. Grill, K.-J. Dietz, N. Amrhein, Z. Naturforsch. C 48 (1993) 867–871.
[101] T. Ishikawa, Trends Biochem. Sci. 17 (1992) 463–468.
[102] Z.-S. Li, Y. Zhao, P.A. Rea, Plant Physiol. 107 (1995) 1257–1268.
[103] M.M.A. Blake-Kalff, J.O.D. Coleman, Planta 200 (1996) 426–431.
[104] A.E. Wolf, K.-J. Dietz, P. Schröder, FEBS Lett. 384 (1996) 31–34.
[105] J.O.D. Coleman, R. Randall, M.M.A. Blake-Kalff, Plant Cell Environ. 20 (1997) 449–460.
[106] S. Farago, C. Brunold, K. Kreuz, Physiol. Plant. 91 (1994) 537–542.
[107] C. Gaillard, A. Dufaud, R. Tommasini, K. Kreuz, N. Amrhein, E. Martinoia, FEBS Lett. 352 (1994) 219–221.
[108] Z.-S. Li, R.-G. Zhen, P.A. Rea, Plant Physiol. 109 (1995) 177–202.
[109] M. Büchler, J. König, M. Brom, J. Kartenbeck, H. Spring, T. Horie, D. Keppler, J. Biol. Chem. 271 (1996) 15091–15098.
[110] R. Tommasini, R. Evers, E. Vogt, C. Mornet, G.J.R. Zaman, A.H. Schinkel, P. Borst, E. Martinoia, Proc. Natl. Acad. Sci. USA 93 (1996) 6743–6748.
[111] J.A.R. Lockhart, A. Samuel, M.P. Greaves, in: R.J. Hance, K. Holly (Eds.), Weed Control Handbook: Principles, Blackwell Scientific Publications, 1989, pp. 43–74.
[112] C.C. Paulusma, P.J. Bosma, G.J. R Zaman, C.T.M. Bakker, M. Otter, G.L. Scheffer, R.J. Scheper, P. Borst, R.P.J.O. Elferink, Science 271 (1996) 1126–1128.
[113] D. Keppler, I. Leier, G. Jedlitschky, Biol. Chem. 378 (1997) 787–791.
[114] K. Sathirakul, H. Susuki, T. Yamada, M. Hanano, Y. Sugiyama, J. Pharmacol. Exp. Ther. 268 (1993) 65–73.
[115] G. Jedlistschky, I. Leier, U. Buchhoz, K. Barnouin, G. Kurz, D. Keppler, Cancer Res. 56 (1996) 988–994.
[116] P. Matile, S. Ginsburg, M. Schellenberg, H. Thomas, Proc. Natl. Acad. Sci. USA 85 (1988) 9529–9532.
[117] B. Hinder, M. Schellenberg, S. Rodoni, S. Ginsburg, E. Vogt, E. Martinoia, P. Matile, S. Hörtensteiner, J. Biol. Chem. 271 (1996) 27233–27236.
[118] M. Wink, Adv. Bot. Res. 25 (1997) 141–169.
[119] M. Klein, G. Weissenböck, A. Dufaud, C. Gaillard, K. Kreuz, E. Martinoia, J. Biol. Chem. 271 (1996) 29666–29671.
[120] M. Klein, E. Martinoia, G. Weissenböck, J. Biol. Chem. 273 (1998) 262–270.
[121] Y. Adachi, H. Kobayashi, Y. Kurumi, M. Shouji, M. Kitano, T. Yamamoto, Hepatology 14 (1991) 655–659.
[122] M. Müller, T. Ishikawa, U. Berger, C. Klünemann, L. Kucka, A. Schreyer, C. Kannich, W. Reutter, G. Kurz, D. Keppler, J. Biol. Chem. 266 (1991) 18920–18926.
[123] T. Nishida, Z. Gatmaitan, M. Che, I.M. Arias, Proc. Natl. Acad. Sci. USA 88 (1991) 6590–6594.
[124] S. Hörtensteiner, E. Vogt, B. Hagenbuch, P.J. Meier, N. Amrhein, E. Martinoia, J. Biol. Chem. 268 (1993) 18446–18449.
[125] D.T. Clarkson, C. Brownles, S.M. Ayling, J. Cell Sci. 91 (1988) 71–80.
[126] S. Gilroy, W.A. Hughes, A.J. Trewavas, Plant Physiol. 90 (1989) 482–491.
[127] L. Cole, J. Coleman, A. Kearns, G. Morgan, C. Hawes, J. Cell Sci. 99 (1991) 545–555.
[128] K.M. Wright, K.J. Oparka, J. Exp. Bot. 45 (1994) 35–44.
[129] M. Klein, E. Martinoia, G. Weissenböck, FEBS Lett. 420 (1997) 86–92.
[130] D. Brauer, J. Otto, S.-I. Tsu, J. Plant Physiol. 145 (1995) 57–61.
[131] M.V. St-Pierre, S. Ruetz, L.F. Epstein, P. Gros, I.M. Arias, Proc. Natl. Acad. Sci. USA 91 (1994) 9476–9479.
[132] D.F. Ortiz, M.V. St-Pierre, A. Abdulmessih, I.M. Arias, J. Biol. Chem. 272 (1997) 15358–15365.
[133] S.S. Strautnieks, L.N. Bull, A.S. Knisely, S. Kocoshis, N. Dahl, H. Arnell, E. Sokal, K. Dahan, S. Childs, V. Ling, M.S. Tanner, A.F. Kagalwalla, A. Nemeth, J. Pawlowska, A. Baker, G. MieliVergani, N.B. Freimer, R.M. Gardiner, R.J. Thompson, Nat. Genet. 20 (1998) 233–238.
[134] K.A. Marrs, M.R. Alfeniot, A.M. Lloyd, V. Walbot, Nature 375 (1995) 397–400.
[135] M.R. Alfenito, E. Souer, C.D. Goodman, R. Buell, J. Mol, R. Koes, V. Walbot, Plant Cell 10 (1998) 1135–1149.
[136] W. Hopp, H.U. Seitz, Planta 170 (1987) 74–85.
[137] E. Grotewold, M. Chamberlin, M. Snook, B. Siame, L. Butler, J. Swenson, S. Maddock, G. St. Clair, B. Bowen, Plant Cell 10 (1998) 721–740.
[138] Z.-S. Li, M. Alfenito, P.A. Rea, V. Walbot, R.A. Dixon, Phytochemistry 45 (1997) 689–693.
[139] M. Urban, T. Bhargarva, J.E. Hamer, EMBO J. 18 (1999) 512–521.
[140] P. Talalay, M. De Long, H.J. Prochaska, Proc. Natl. Acad. Sci. USA 85 (1998) 8261–8265.
[141] K.A. Marrs, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 127–158.
[142] M.M.A. Blake-Kalff, R.A. Randall, J.O.D. Coleman, in: K.K. Hatzios (Ed.), Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants, Kluwer Academic Publishers, Utrecht, 1997, pp. 245–259.
[143] J.-P. Galaud, M. Carrière, N. Pauly, H. Canut, P. Chalon, D. Caput, R.F. Pont-Lezica, Plant J. 17 (1999) 111–118.
[144] M.P. Anderson, R.J. Gregory, S. Thompson, D.W. Souza, P. Sucharita, R.C. Mulligan, A.E. Smith, M.J. Welsh, Science 253 (1991) 202–205.
[145] C.E. Bear, C. Li, N. Kartner, R.J. Bridges, T.J. Jensen, M. Ramjeesingh, J.R. Riordan, Cell 68 (1992) 809–818.
[146] N.A. McCarty, S. McDonough, B.N. Cohen, J.R. Riordan, N. Davidson, H.A. Lester, J. Gen. Physiol. 102 (1993) 1–23.
[147] M. Egan, T. Flotte, S. Afione, R. Solow, P.L. Zeitlin, B.J. Carter, W.B. Guggino, Nature 358 (1993) 581–584.

[148] B.R. Grubb, A.M. Paradiso, R.C. Boucher, Am. J. Physiol. 267 (1994) C293–C300.
[149] T.C. Chinet, J.M. Fulton, J.L. Yarkakas, R.C. Boucher, M.J. Stutts, Am. J. Physiol. 266 (1994) C1061–C1068.
[150] M.A. Valverde, J.A. O'Brien, F.V. Sepulveda, R.A. Ratcliff, M.J. Evans, W.H. Colledge, Proc. Natl. Acad. Sci. USA 92 (1995) 9038–9041.
[151] C.M. McNicholas, W.B. Guggino, E.M. Schiebert, S.C. Hbert, G. Giebisch, M.E. Egan, Proc. Natl. Acad. Sci. USA (1996) 8083–8088.
[152] A. Ishida-Takahashi, H. Otani, C. Takahashi, T. Washizuka, K. Tsuji, M. Noda, M. Horie, S. Sasayama, J. Physiol. 508 (1998) 23–30.
[153] N. Inagaki, T. Gonoi, J.P. Clement, N. Namba, J. Inazawa, G. Gonzalez, L. Aguilar-Bryan, S. Seino, J. Bryan, Science 270 (1995) 1166–1170.
[154] M. Schwanstecher, C. Sierverding, H. Dorschner, I. Gross, L. Aguilar-Bryan, C. Schwanstecher, J. Bryan, EMBO J. (1998) 5529–5535.
[155] D.N. Sheppard, M.J. Welsh, J. Gen. Physiol. 100 (1992) 573–591.
[156] B.D. Schulz, A.D.G. Deroos, C.J. Venglarik, A.K. Singh, R.A. Frizzell, R.J. Bridges, Am. J. Physiol. 15 (1996) L192–L200.
[157] J. Jirsch, R.G. Deeley, S.P.C. Cole, A.J. Stewart, D. Fedida, Cancer Res. 53 (1993) 4156–4160.
[158] M.A. van Kuijck, R.A.M.H. van Aubel, A.E. Busch, F. Lang, F.G.M. Russel, R.J.M. Bindels, C.H. van Os, P.M.T. Deen, Proc. Natl. Acad. Sci. USA 93 (1996) 5401–5406.
[159] S.M. Assmann, Annu. Rev. Cell Biol. 9 (1993) 345–375.
[160] C. Schmidt, I. Schelle, Y.J. Liao, J.I. Schroder, Proc. Natl. Acad. Sci. USA 92 (1995) 9535–9539.
[161] B. Schulz-Lessdorf, G. Lohse, R. Hedrich, Plant J. 10 (1996) 993–1004.
[162] N. Leonhardt, E. Marin, A. Vavasseur, C. Forestier, Proc. Natl. Acad. Sci. USA 94 (1997) 14156–14161.
[163] W.H. Wu, S.M. Assmann, Plant Physiol. 107 (1995) 101–109.
[164] K. Czempinski, S. Zimmermann, T. Ehrhardt, B. Müller-Röber, EMBO J. 16 (1997) 2565–2575.
[165] F. Gaymard, G. Pilot, B. Lacombe, D. Bouchez, D. Bruneau, J. Boucherez, N. MichauxFerriere, J.B. Thibaud, H. Sentenac, Cell 94 (1998) 647–655.
[166] J.I. Schroeder, R. Hedrich, J.M. Fernandez, Nature 312 (1984) 361–362.
[167] R.L. Nakamura, J.A. Anderson, R.F. Gaber, Plant Physiol. 109 (1995) 371–374.
[168] B. Müller-Röber, J. Ellenberg, N. Provart, L. Willmitzer, H. Busch, D. Becker, S. Hoth, R. Hedrich, EMBO J. 14 (1995) 2409–2416.
[169] J.I. Schroeder, S. Hagiwara, Nature 338 (1989) 427–430.
[170] R. Hedrich, H. Busch, K. Raschke, EMBO J. 9 (1990) 3889–3892.
[171] N. Leonhardt, A. Vavasseur, C. Forestier, Plant Cell 11 (1999) 1141–1151.
[172] M.A. Valverde, M. Diaz, F.V. Sepulveda, D.R. Gill, S.C. Hyde, C.F. Higgins, Nature 355 (1992) 830–833.
[173] D.R. Gill, S.C. Hyde, C.F. Higgins, M.A. Valverde, G.M. Mintenig, F.V. Sepulveda, Cell 71 (1992) 23–32.
[174] G.M. Mintenig, M.A. Valverde, F.V. Sepulveda, D.R. Gill, S.C. Hyde, J. Kirk, C.F. Higgins, Recept. Channels 1 (1993) 305–313.
[175] S.P. Hardy, H.R. Goodfellow, M.A. Valverde, D.R. Gill, F.V. Sepúlveda, C.F. Higgins, EMBO J. 14 (1995) 68–75.
[176] H.R. Goodfellow, A. Sardini, S. Ruetz, R. Callaghan, P. Gros, P.A. McNaughton, C.F. Higgins, J. Biol. Chem. 271 (1996) 13668–13674.
[177] C.C. Smart, A.J. Trewavas, Plant Cell. Environ. 6 (1983) 507–514.
[178] K. Chloupková, C.C. Smart, Plant Physiol. 105 (1984) 497–507.
[179] A. Kralli, S.P. Bohen, K.R. Yamamoto, Proc. Natl. Acad. Sci. USA 92 (1995) 4701–4705.
[180] W. Schuster, A. Brennicke, Annu. Rev. Plant Physiol. Plant Mol. Biol. 45 (1994) 61–78.
[181] R. Kranz, R. Lill, B. Goldman, G. Bonnard, S. Merchant, Mol. Microbiol. 29 (1998) 383–396.
[182] B.S. Goldman, D.L. Beckman, A. Bali, E.M. Monika, K.K. Gabbert, R.G. Kranz, J. Mol. Biol. 268 (1997) 724–738.
[183] W. Schuster, B. Combettes, K. Flieger, A. Brennicke, Mol. Gen. Genet. 239 (1993) 49–57.
[184] S. Theg, S.M. Theg, J. Cell. Biochem. 17C (Suppl.) (1993) 17.
[185] P. Matile, Adv. Bot. Res. 25 (1997) 87–112.
[186] R.H. Valdivia, L. Wang, S.C. Winans, J. Bacteriol. 173 (1991) 6398–6405.
[187] H. Zanker, L.J. Von Lintig, J. Schroder, J. Bacteriol. 174 (1992) 841–849.
[188] G.T. Hayman, S. Beck von Bodman, H. Kim, P. Jiang, S. Farrand, J. Bacteriol. 175 (1993) 5575–5584.
[189] T. Dylan, L. Ielpi, S. Stanfield, L. Kashyap, C. Douglas, M. Yanofsky, E. Nester, D.R. Helsinki, G. Ditta, Proc. Natl. Acad. Sci. USA 83 (1986) 4403–4407.
[190] S.W. Stanfield, L. Ielpi, D. O'Brochta, D.R. Helsinki, G.S. Ditta, J. Bacteriol. 170 (1988) 3523–3530.
[191] G.A. Cangelosi, G. Martinetti, J.A. Leigh, C.C. Lee, C. Theines, E.W. Nester, J. Bacteriol. 171 (1989) 1609–1615.
[192] A.K. Scheu, A. Economou, G.F. Hong, S. Ghelani, A.W.B. Johnston, J.A. Downie, Mol. Microbiol. 6 (1992) 231–238.
[193] J. Król, A. Skorupska, Microbiology 143 (1997) 1389–1394.
[194] S. Bardin, S. Dan, M. Osteras, T.M. Finan, J. Bacteriol. 178 (1996) 4540–4547.
[195] M. Rosenblueth, M.F. Haynes, E. Martínez-Romero, Mol. Gen. Genet. 258 (1998) 587–598.

[196] M. Sami, H. Yamashita, T. Hirono, H. Kadokura, K. Kitamoto, K. Yoda, M. Yamasaki, J. Ferment. Bioeng. 84 (1997) 1–6.
[197] C. Bayliss, E. Bent, D.E. Culham, S. Maclellan, A.J. Clarke, G.L. Brown, J.M. Wood, Can. J. Microbiol. 43 (1997) 809–818.
[198] W.B. Frommer, O. Ninnemann, Annu. Rev. Plant Physiol. Plant Mol. Biol. 46 (1995) 419–444.
[199] L. Covic, R.R. Lew, Biochim. Biophys. Acta 1305 (1996) 125–129.
[200] D.R. Nelson, L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyereisen, D.J. Waxman, M.W. Waterman, O. Gotoh, M.J. Coon, R.W. Estabrook, I.C. Gunsalus, D.W. Nebert, Pharmacogenetics 6 (1996) 1–42.
[201] http://drneslson.utmem.edu/CytochromeP450.html

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Structure of an *mdr*-like Gene from *Arabidopsis thaliana*

EVOLUTIONARY IMPLICATIONS*

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Multidrug resistance of mammalian tumor cells is caused by the enhanced expression of P-glycoproteins. These proteins are encoded by *mdr* genes and mediate the energy-dependent efflux of a variety of lipophilic drugs from cells. To test whether in plants *mdr*-like genes might be involved in certain cases of cross-resistance to different herbicides, we have cloned and characterized a gene from *Arabidopsis thaliana*, *atpgp1*, encoding a putative P-glycoprotein homologue. Like the mammalian P-glycoproteins, with which it shares extensive sequence homology and a similar organization in structural domains, this protein is internally duplicated. Seven of the nine introns in the *atpgp1* gene match introns in the mammalian *mdr* genes to within a few nucleotides, and the positions of these suggest that P-glycoprotein genes evolved by duplication and subsequent fusion of an intron-containing primordial gene prior to the evolutionary separation of plants and mammals. The *atpgp1* gene gives rise to transcripts present in all plant parts but particularly abundant in inflorescence axes.

Multidrug resistance (MDR)[1] is a phenomenon described in mammalian tumor cells that hampers chemotherapy of cancer. Cells initially sensitive to a cytotoxic drug can evolve into a state in which they are not only resistant against the same drug but also against a wide variety of structurally unrelated drugs (see Endicott and Ling (1989), Van der Bliek and Borst (1989), and Pastan and Gottesman (1991) for reviews). Multidrug-resistant cells are characterized by a decreased intracellular drug accumulation that is often caused by the increased expression of ATP-driven drug efflux pumps, the P-glycoproteins. These membrane-bound proteins are encoded by a small group of closely related *mdr* genes (Chen *et al.*, 1986; Gros *et al.*, 1986; Van der Bliek *et al.*, 1987; Croop *et al.*, 1989; Ng *et al.*, 1989) and belong to the superfamily of ATP-binding cassette-containing transport proteins (Hyde *et al.*, 1990). Proteins in this superfamily include components of the high affinity uptake systems, as well as certain export systems of bacteria and share a highly conserved nucleotide-binding domain (Higgins *et al.*, 1986; Ames, 1986).

Apart from mammals, P-glycoprotein-like genes are associated with a drug resistance resembling MDR in *Plasmodium falciparum*, where a gene called *pfmdr* encoding a putative P-glycoprotein homologue appears to mediate resistance to chloroquine (Foote *et al.*, 1989), and in *Entamoeba histolytica*, where *mdr*-like sequences are overexpressed in emetine-resistant mutants (Samuelson *et al.*, 1990). Furthermore, in *Leishmania tarentolae*, the P-glycoprotein gene *ltpgpA* is located on amplified H circles in methotrexate-resistant strains, although it is not yet clear how the gene may be involved in resistance (Ouellette *et al.*, 1990).

MDR-like phenomena have not been described in plants. However, recently the development of cross-resistance to herbicides in weeds is becoming more common (Putwain and Collin, 1989). Whereas cross-resistance to herbicides with a similar site and mode of action may be readily explained by mutation of the target site, there are reports of cross-resistance that seem inexplicable because they involve herbicides of different structure, site, and mode of action (*e.g.* Heap and Knight, 1986; Moss and Cussans, 1987). To test whether P-glycoprotein-like molecules are involved in such instances of cross-resistance, we set out to clone possible P-glycoprotein homologues in plants. Here we report the isolation and complete sequence of a gene from *Arabidopsis thaliana* that is homologous to the mammalian *mdr* genes, and we discuss the evolutionary implications of the intron/exon structure of this gene for the origin of P-glycoproteins.

EXPERIMENTAL PROCEDURES

Library Screening—As hydridization probes for the library screening, three pools of oligonucleotides were synthesized encoding three amino acid sequences in the neighborhood of the highly conserved second nucleotide-binding fold that are identical between the *Escherichia coli hlyB* protein (Felmlee *et al.*, 1985), and N-terminal and C-terminal halves of the human *MDR1* product (Chen *et al.*, 1986). Probe 1 contained all 384 possible 17-mers encoding the amino acid sequence SGGQKQ, probe 2 all 864 17-mers encoding the sequence IAIARA, and probe 3 all 385 17-mers coding for the sequence DEATSA, the wobble base of the codon encoding the C-terminal amino acid always being omitted. These pools were 5′ end-labeled to high specific activity with [γ-^{32}P]ATP (6,000 Ci/mmol; Du Pont-New England Nuclear) and polynucleotide kinase according to standard procedures (Maniatis *et al.*, 1982). About 300,000 clones of a commercially available genomic library of *A. thaliana* L. Columbia) constructed in λEMBL3 (Clontech Laboratories Inc., Palo Alto, CA) were plated, and triplicate sets of filters were taken. To minimize the influence of base composition on the hybridization temperature, hybridization was carried out in buffer containing 3 M tetramethylammonium chloride (Wood *et al.*, 1985), 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, 2% sodium lauroyl sarcosinate at 50 °C overnight. Washing was performed in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate), 1% sodium lauroyl sarcosinate at 37 °C. Rescreening of clones giving a signal with all three probes resulted in eight positive candidates that were purified. Hybridization of oligonucleotide pool 3 to a Southern blot containing DNA of these clones digested with *Hin*dIII, *Pst*I, and *Bam*HI suggested that the clones could be divided into two classes according to their restriction pattern.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X61370.

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[1] The abbreviations used are: MDR, multidrug resistance; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction.

A hybridizing 1.4-kb *Hin*dIII fragment that was characteristic for one of the classes (corresponding to *probe 1* in Fig. 1) was subcloned into pBluescript SK+ (Stratagene, La Jolla, CA), sequenced, and found to have homology on the amino acid level to mammalian *mdr* genes. This fragment was used to rescreen the Southern blot, and one clone, λatpgp16, that contained hybridizing *Pst*I fragments of 4 and 5 kb length (Fig. 1), was chosen for further analysis. The 4- and 5-kb *Pst*I fragments were subcloned into pBluescript (KS+ and SK+, respectively) and used for sequence analysis in addition to the subcloned 1.4-kb *Hin*dIII fragment.

Sequence Analysis—The sequence of the 6.3-kb stretch of DNA shown in Fig. 2 was determined completely on both strands by the chain termination method (Sanger *et al.*, 1977) using overlapping deletion fragments created by exonuclease III as double stranded templates. Gaps were filled in by using suitable synthetic oligonucleotides as primers. Sequencing reactions were performed with Sequenase (U. S. Biochemical Corp., Cleveland, OH) according to the instructions of the manufacturer. Sequences were assembled and analyzed using the Staden Plus (Amersham International PLC, Amersham, UK) and PC/GENE (IntelliGenetics, Mountain View, CA) computer software. The intron/exon structure was confirmed by comparison of cDNA and gene sequences.

cDNA Cloning—Amplification by the polymerase chain reaction (PCR) of cDNAs was accomplished essentially as described by Kawasaki and Wang (1989). Briefly, 25 μg of LiCl-precipitated RNA extracted from peduncles was denatured at 90 °C for 2 min and subsequently reverse-transcribed in a volume of 100 μl containing 10 μl of 10 × PCR buffer (1 × PCR buffer: 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg/ml nuclease-free bovine serum albumin), 1 mM dNTP, 100 units of RNasin, 500 pmol of random hexamer oligonucleotides, and 1000 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) for 10 min at room temperature and for an additional 60 min at 37 °C. After heat inactivation at 90 °C for 3 min, aliquots of 10 μl were amplified with each of six pairs of primers in a final volume of 100 μl supplemented with 9 μl of 10 × PCR buffer, 8 μl of 1.25 mM dNTP, 2.5 units of Taq polymerase (Perkin-Elmer Cetus Instruments) and 100 pmol of each primer oligonucleotide through 50 cycles using a thermal cycler (Perkin-Elmer Cetus Instruments) and the following cycle parameters: denaturation for 1 min at 94 °C, primer annealing for 30 s at 51 °C, and extension for 1 min at 72 °C. The amplification primers corresponded to the sequences in (originally presumed) exons marked by *stippled arrows* in Fig. 2 but contained additional nucleotides at their 5′ ends that created suitable restriction sites to facilitate cloning into pBluescript. Thus, six overlapping partial cDNAs were obtained that together comprised the complete coding sequence.

DNA and RNA Extraction—For DNA isolation, leaf tissue of *A. thaliana* L. Columbia was homogenized in liquid N_2, suspended in 5 volumes of lysis buffer (7 M urea, 350 mM NaCl, 50 mM Tris-HCl, pH 8, 20 mM EDTA, 1% sodium lauroyl sarcosinate), and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase by the addition of 0.1 volume of 3 M sodium acetate and 1 volume of ethanol. The precipitate was redissolved in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), run through a CsCl/EtBr gradient according to standard procedures, and dialyzed against TE.

RNA from various tissues was isolated by the hot phenol method. The tissue was powdered in liquid nitrogen and added immediately to a 1:1 mixture of phenol and 2 × NETS (200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate) preheated to 80 °C. The mixture was thoroughly vortexed, cooled on ice, and centrifuged. After two more phenol extractions at room temperature, the nucleic acids were precipitated with ethanol and redissolved in water. RNA was then precipitated by adding 1 volume of 5 M LiCl and incubating on ice for 4 h. The RNA was pelleted, dissolved in water, and used for PCR amplification, S1 mapping, and Northern analysis.

S1 Mapping and Northern and Southern Hybridizations—For S1 mapping, a 540-nucleotide-long continuously labeled single-stranded probe that was complementary to the sense strand from position 709 to 1109 (the rest being derived from vector sequences) was synthesized from a double-stranded deletion template using one of the amplification primers (5′ attctAGAGCAGCCATGGACAAAAGC; the sequence given in capitals is complementary to the sense strand from position 1089 to 1109 (Fig. 2); lower case letters denote additional nucleotides creating an *Xba*I restriction site) in a sequencing reaction in the absence of dideoxynucleotides and the presence of [α-^{32}P] dCTP (6000 Ci/mmol; Du Pont-New England Nuclear). The reaction was run on a sequencing gel, from which the probe was cut out and eluted. S1 mapping was performed with 10 μg of LiCl-precipitated RNA as previously described (Dudler and Travers, 1984), except that 220 units of S1 nuclease (Boehringer Mannheim) were used per reaction. A sequencing reaction using the same template and primer was run in parallel as a marker. Note, however, that S1 nuclease is expected to remove the 5 noncomplementary nucleotides at the 5′ end of the oligonucleotide-primed probe.

For Southern and Northern analysis, DNA gels and RNA formaldehyde gels were blotted onto GeneScreen membranes (Du Pont-New England Nuclear) and hybridized to random-primed ^{32}P-labeled probes according to standard procedures (Maniatis *et al.*, 1982). The probe for genomic Southern and Northern blot hybridizations was a 2.2-kb fragment (Fig. 1; *probe 2*) bounded by a *Hin*dIII site (starting at position 3433) at the left and a deletion break point (position 5582; cut with *Kpn*I at a neighboring site in the vector) at the right side.

RESULTS

Isolation and Analysis of a P-glycoprotein Gene of A. thaliana—To clone possible *mdr* homologues of *A. thaliana*, three pools of degenerate oligonucleotides were synthesized corresponding to three amino acid sequences around the second nucleotide-binding fold that are conserved in mammlian P-glycoproteins and the *hlyB* gene product of *E. coli*. A λEMBL3 genomic library of *A. thaliana* was screened, and clones hybridizing with all three oligonucleotide pools were isolated. The positive clones could be divided into two classes according to their restriction and probe hybridization pattern, suggesting that each class represented overlapping fragments of two different genomic loci. Sequence determination of a 1.4-kb *Hin*dIII fragment that was characteristic for one class of clones and that hybridized to the oligonucleotide probes revealed sequence similarity to mammalian *mdr* genes at the amino acid level. Consequently, one clone of this class, λatpgp16, was chosen for further analysis. Two *Pst*I insert fragments of 4 and 5 kb that both hybridized to the 1.4-kb *Hin*dIII fragment (Fig. 1, *probe 1*) were subcloned and sequenced in part. Analysis of the resulting 6300-bp sequence shown in Fig. 2 suggested that it contained a gene with nine introns and 10 exons encoding a protein of 1286 amino acids. Structure and exon sequences of the gene were confirmed by sequencing cDNA clones covering the complete coding region. Since screening of an *A. thaliana* cDNA library with the 1.4-kb *Hin*dIII fragment was not successful, single-stranded cDNA was prepared from RNA extracted from peduncles (see below) and amplified by the polymerase chain reaction with six pairs of suitable primers (Fig. 2, *stippled arrows*). Thus, six overlapping cDNA fragments were obtained covering exon sequences from nucleotide positions 793 to 5853. The sequence of these cDNAs was in agreement with the gene structure shown in Fig. 2.

The transcription initiation site was determined by S1 mapping. A continuously labeled oligonucleotide-primed single-stranded probe was synthesized that was complementary to the sense strand from nucleotide positions 709 to 1109 (Fig. 2). As shown in Fig. 3, the protected fragment of about 315 nucleotides co-migrated approximately with the C-band (po-

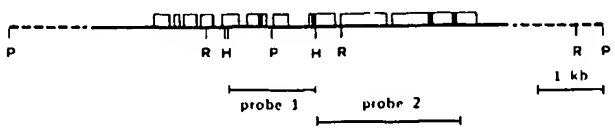

FIG. 1. **Physical map of the *atpgp1* gene of *A. thaliana*.** The *thick solid line* represents sequenced DNA; *stippled lines* denote regions not sequenced. *Open boxes* indicate exons, with the *black bars* marking the nucleotide-binding folds. *Thin lines* represent probe fragments used for hybridization studies and a *scale bar*. *P*, *Pst*I; *R*, *Eco*RI; *H*, *Hin*dIII.

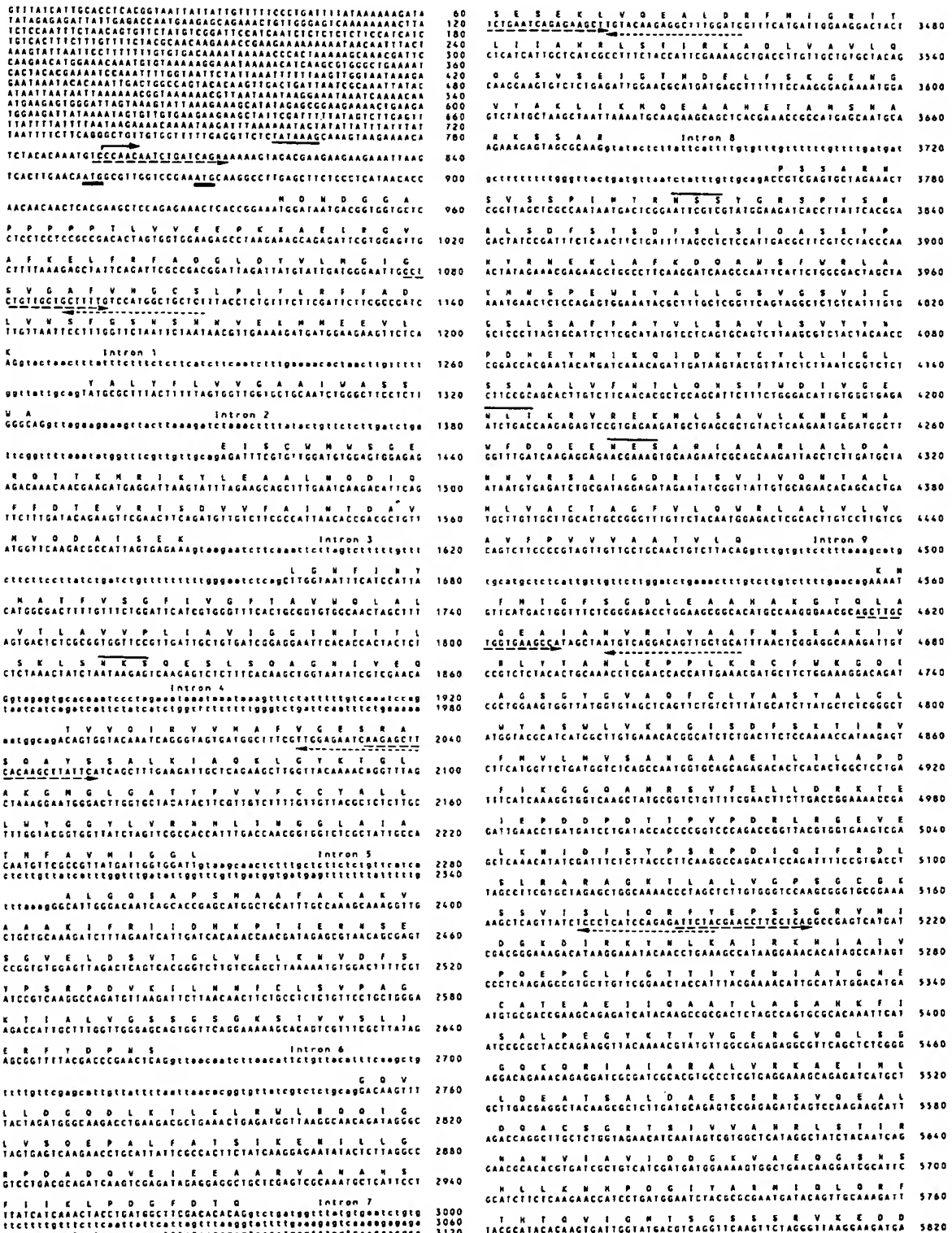

FIG. 2. **Nucleotide sequence of the *atpgp1* gene and deduced amino acid sequence of the encoded product.** Intron sequences are given in *lowercase letters*. The amino acid sequence is written in the *single letter code* above exon sequences. The presumed TATA box is *underlined*, and the *solid arrow* indicates the site of

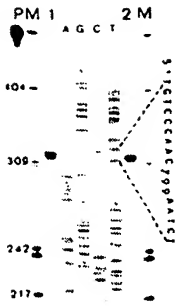

FIG. 3. **S1 mapping of the *atpgp1* transcript.** The probe fragment protected using 10 μg of total RNA extracted from peduncles is shown in *lanes 1* and *2*. *Lane P*, full length S1 probe. *Lane M*, marker (*Hpa*II-cut pBR322). The *numbers* indicate the length in nucleotides of the marker fragments. *Lanes A, G, C,* and *T*, sequence ladder generated with the same primer as the S1 probe. The sequence of the region around the C at position 799 co-migrating with the protected probe fragment is given on the *right*.

sition 799) on the sequence ladder primed with the same oligonucleotide. Since this oligonucleotide had 5 noncomplementary residues at its 5′ end (comprising a restriction site) that were cleaved off by S1 nuclease, the transcription start site maps, in effect, 5 nucleotides upstream, that is around the C at position 794 (Fig. 2, *arrow*). A potential TATA box (*underlined* in Figure 2) is located 28 bp upstream of this position.

The assignment of the translation initiation site was somewhat problematic. The most upstream in-frame potential initiation codon in the 3855-bp-long open reading frame following it in the cDNA sequence (corresponding to position 939 in Fig. 2) is preceded by two out-of-frame methionine codons at positions 852 and 868, respectively (Fig. 2, *bars*). However, since these are followed by termination codons after only 27 and 111 nucleotides, respectively, we tentatively assigned the methionine codon starting at position 939, which exhibits a reasonable match to the consensus initiation sequence for dicot plants (Cavener and Ray, 1991), to be the translation initiation site. The resulting putative translation product of 1286 amino acids with a calculated relative molecular mass of 140,574 is of length similar to that of the mammalian P-glycoproteins, which are around 1280 amino acids long (Chen *et al.*, 1986; Gros *et al.*, 1986; Van der Bliek *et al.*, 1987). Like the latter proteins, it is composed of two homologous halves, the N-terminal part (amino acids 1–630) sharing 35% identical and 16% conserved amino acids with the C-terminal part (amino acids 631–1286). Its hydropathy profile (not shown) is similar to that of the mammalian P-glycoproteins and exhibits six hydrophobic regions in each protein half that are thought to be membrane spanning. When calculated with the HELIXMEM program of PC/GENE (based on the algorithm of Eisenberg *et al.*, 1984), only 11 membrane-associated helices were indicated (*underlined* in Fig. 6*a*), although the region corresponding to the missing one (the third in the C-terminal half) is hydrophobic and shows sequence conservation with the corresponding regions of the mammalian P-glycoproteins. As in the latter proteins, highly conserved nucleotide-binding consensus sequences (Higgins *et al.*, 1986; *boxed* in Fig. 6*A*) are present in both protein halves, and there are several potential *N*-glycosylation sites (*overlined* in Fig. 2). Sequence comparison with other P-glycoproteins revealed that the putative *Arabidopsis* protein shares 39% identical and 16% conserved amino acids with the products of the human *MDR1* and *MDR3* genes (Chen *et al.*, 1986; Van der Bliek *et al.*, 1987) and the mouse *mdr1* gene (Gros *et al.*, 1986). As is evident from the dot matrix comparison of the *Arabidopsis* protein and the human *MDR1* P-glycoprotein presented in Fig. 4, the sequence similarity extends essentially over the complete sequence, although it is highest in the regions containing the nucleotide-binding folds (amino acids 390–630 and 1040–1280). In contrast, sequence similarity to nonmammalian P-glycoproteins is much more confined to the nucleotide-binding domains and therefore less pronounced (*e.g.* 24% overall identity with both the yeast *STE6* gene product (McGrath and Varshavsky, 1989) and the *P. falciparum* P-glycoprotein (Foote *et al.*, 1989) and 15% with the *L. tarentolae* P-glycoprotein (Ouellette *et al.*, 1990)). From this analysis, we conclude that we have isolated a gene encoding a P-glycoprotein homologue of *A. thaliana*. We name this gene *atpgp1*.

The result of a genomic Southern hybridization using probe 2 (Fig. 1) with moderately stringent washing conditions (2 × SSC, 60 °C) is shown in Fig. 5*A*. Whereas the hybridization pattern obtained with *Hin*dIII- and *Bam*HI-digested DNA is compatible with *atpgp1* being a single copy gene, the pattern obtained with *Eco*RI-restricted DNA indicated additional homologous sequences. Of the four bands visible with *Eco*RI-restricted DNA, only the 3.5- and 2.2-kb bands correspond to the cloned *atpgp1* region, as is evident from the restriction map shown in Fig. 1. (The 2.2-kb band is expected to be weaker than the 3.5 kb one because it is covered by the probe to a lesser extent.) Since at least the smaller one of the additional bands cannot be attributed to incomplete DNA restriction (being smaller than the expected bands), the hybridization pattern indicates a second cross-hybridizing P-glycoprotein-like gene. Additional evidence for a second gene comes also from our library screening experiments, which resulted in two classes of positive clones. Indeed, preliminary partial sequence analysis of a member of the second class revealed equal similarity to both the *atpgp1* and the mammalian *mdr* genes.[2] Thus, the *A. thaliana* genome seems to harbor at least another, possibly quite diverged, *mdr*-like gene in addition to *atpgp1*.

Expression of the atpgp1 Gene—The S1 mapping experiment and the complete agreement of cDNA and gene sequences indicate that the *atpgp1* gene is expressed. Northern analysis using probe 2 (Fig. 1) revealed that RNA homologous to the *atpgp1* gene with a size of about 4.5 kb is present in all plant parts examined, *i.e.* leaves, roots, flower buds, open flowers, and peduncles (Fig. 5*B*, inflorescence axes). In the latter organ, however, it is about 5 times more abundant per equivalent of total RNA than in all other tissues, suggesting that the *atpgp1* gene may be expressed in a tissue or cell type-specific manner. The size of the hybridizing mRNA is compatible with the minimum size of 4 kb expected from the cDNA sequence, which did not include the 3′-noncoding region. It is not known whether the smaller band visible in all lanes in Fig. 5*B* represents a degradation product of the

[2] R. Dudler and C. Hertig, unpublished results.

transcription initiation. Two out-of-frame methionine codons upstream of the presumed translation initiation site are *underlined* with *thick bars*. Potential *N*-glycosylation sites are *overlined* in the protein sequence. *Stippled arrows* indicate the oligonucleotides used as primers for PCR amplification of cDNAs. The *right-pointing ones* underline the sequences of the upstream primers, the *left-pointing ones* underline the complementary sequences of the downstream primers. Each of the six upstream primers was used with its nearest downstream primer.

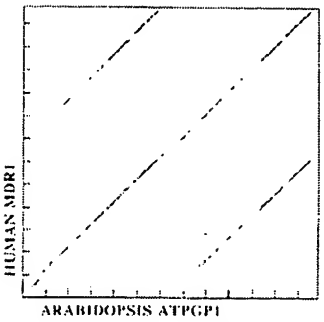

FIG. 4. **Dot matrix comparison of the human *MDR1* P-glycoprotein (Chen *et al.*, 1986) and the *Arabidopsis atpgp1* gene product.** The proportional algorithm of the DIAGON program (Staden Plus software) was used with a window length of 19 and a score of 228. *Marks* on the *axes* denote intervals of 100 amino acids.

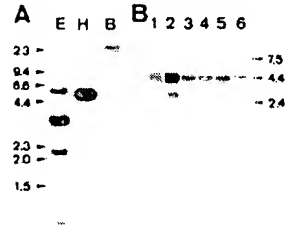

FIG. 5. **Southern and Northern analyses with an *atpgp1* probe.** *A*, autoradiogram of a Southern blot hybridized with probe 2. Each lane contained 10 μg of genomic *Arabidopsis* DNA restricted with *Eco*RI (*E*), *Hin*dIII (*H*), and *Bam*HI (*B*). *Numbers* on the *left* give the size in kb of marker bands. *B*, autoradiogram of a Northern blot hybridized with probe 2. Each lane contained 10 μg of total RNA extracted from leaves (*lanes 1* and *3*), peduncles (*lane 2*), flower buds (*lane 4*), open flowers (*lane 5*), and roots (*lane 6*). *Numbers* on the *right* indicate the size in kb of RNA marker bands.

4.5-kb RNA or a genuine different species, originating from the same or another gene.

Comparison of Intron Positions—The mammalian *mdr* genes sequenced to date contain 28 introns, 26 of which interrupt the coding region (Raymond and Gros, 1989; Chen *et al.*, 1990; Lincke *et al.*, 1991). Thus, it was interesting to compare the intron positions of the *atpgp1* and the mammalian *mdr* genes. For the present purpose, the nine introns in *atpgp1* are referred to as A1–A9 and the 26 introns in the protein coding regions of the mammalian *mdr* genes, as M2–M27. Fig. 6*A* shows an alignment of N-terminal and C-terminal halves of P-glycoproteins encoded by the *Arabidopsis atpgp1*, the human *MDR*1, and mouse *mdr*1 genes. The intron positions relative to the protein sequences (*arrowheads*) and intron phases are indicated. As is evident, of the eight introns located in the left half of the *Arabidopsis* gene, none matches the position of the ninth in the right half. Furthermore, none of the nine introns in the *Arabidopsis* gene exactly matches any of the 26 introns located in the protein coding regions of the mammalian *mdr* genes. However, some are located at similar positions. Within the transmembrane domains, A1 and M5 interrupt the left halves of the P-glycoprotein genes at positions 1 bp apart in the same codon, and hence are of different types (type 0 introns interrupt between codons, type 1 and 2 interrupt after the first and second nucleotide of a codon, respectively (Traut, 1988)). Similarly, A2 in the N-terminal half and M18 in the C-terminal half interrupt corresponding codons and are also of different types. Of the same type are C-terminal A9 and N-terminal M7, which are located at positions two codons apart. Also of the same type but offset by four and five codons are two other possible pairs involving N-terminal *Arabidopsis* and C-terminal mammalian introns, namely A3/M20 and A4/M21. The two *Arabidopsis* introns in the nucleotide-binding domain are located both in the left half. A6 is positioned one and two codons upstream of the closely matched mammalian intron pair M12/M25, respectively, but is of a different type, whereas A7 is located five codons downstream of, and is of the same type as, the conserved mammalian intron pair M13/M26. Of the remaining two *Arabidopsis* introns, A5 does not match any mammalian intron and A8 interrupts the *atpgp1* gene between its left and right halves and hence is located at a position analogous to that of mammalian intron M15.

Thus, seven of the nine introns in the *Arabidopsis atpgp1* gene match mammalian introns in position to within five codons. To clarify the following discussion, a schematic map indicating only the positions of the matched introns is presented in Fig. 6*B*. We note that there are instances of approximate matches between *Arabidopsis* and mammalian introns located both in corresponding (A1/M5), as well as in different gene halves (A2/M18, A9/M7, A3/M20, A4/M21).

DISCUSSION

Evolutionary Implications of Intron Positions—The structural similarity of N-terminal and C-terminal halves of the mammalian P-glycoproteins has been hypothesized to be the result of a duplication and subsequent fusion of an ancestral gene (Chen *et al.*, 1986; Gros *et al.*, 1986). However, although intron positions are conserved in all mammalian P-glycoprotein genes analyzed to date (Raymond and Gros, 1989; Ng *et al.*, 1989; Chen *et al.*, 1990; Lincke *et al.*, 1991), and although both gene halves contain a similar number of introns, an alignment of left and right halves revealed few matches of intron positions between the two halves. This has led to conflicting interpretations concerning the evolution of these genes. Raymond and Gros (1989) preferred the hypothesis that *mdr* genes represent a duplicated intron-containing ancestral gene because at least one intron pair (M13 and M26; Fig. 6*A*) occupies identical positions in both halves of P-glycoprotein genes. In contrast, Chen *et al.* (1990) proposed that P-glycoprotein genes resulted from the fusion of two independently evolved genes encoding the two halves. These two primordial genes would have been formed separately by the fusion of closely related sequences encoding the nucleotide-binding domain with sequences coding for different transmembrane proteins. Chen *et al.* based their proposal on the observation that both the sequence and the intron/exon-structure are much more conserved between the regions encoding the nucleotide-binding domains of the two gene halves than in the regions encoding the transmembrane domains.

How can the intron positions in the *atpgp1* gene be interpreted in the light of these two conflicting hypotheses? As outlined above, seven of the nine *Arabidopsis* introns match mammalian introns in position to within five codons (Fig. 6*B*). Although these approximate matches could in principle be purely coincidental, we think it is more plausible to assume that they are derived from exact matches by intron sliding, even though in three of the seven pairs the intron type is not conserved. With this assumption, the most straightforward interpretation of the fact that there are matches between plant and mammalian introns located both in corresponding (A1/M5) and in different gene halves (A2/M18, A9/M7, A3/M20, A4/M21; Fig. 6*B*), is that both gene halves arose by duplication of a primordial gene in a common ancestor to plants and mammals. This primordial gene would have contained at least seven introns at positions where introns located

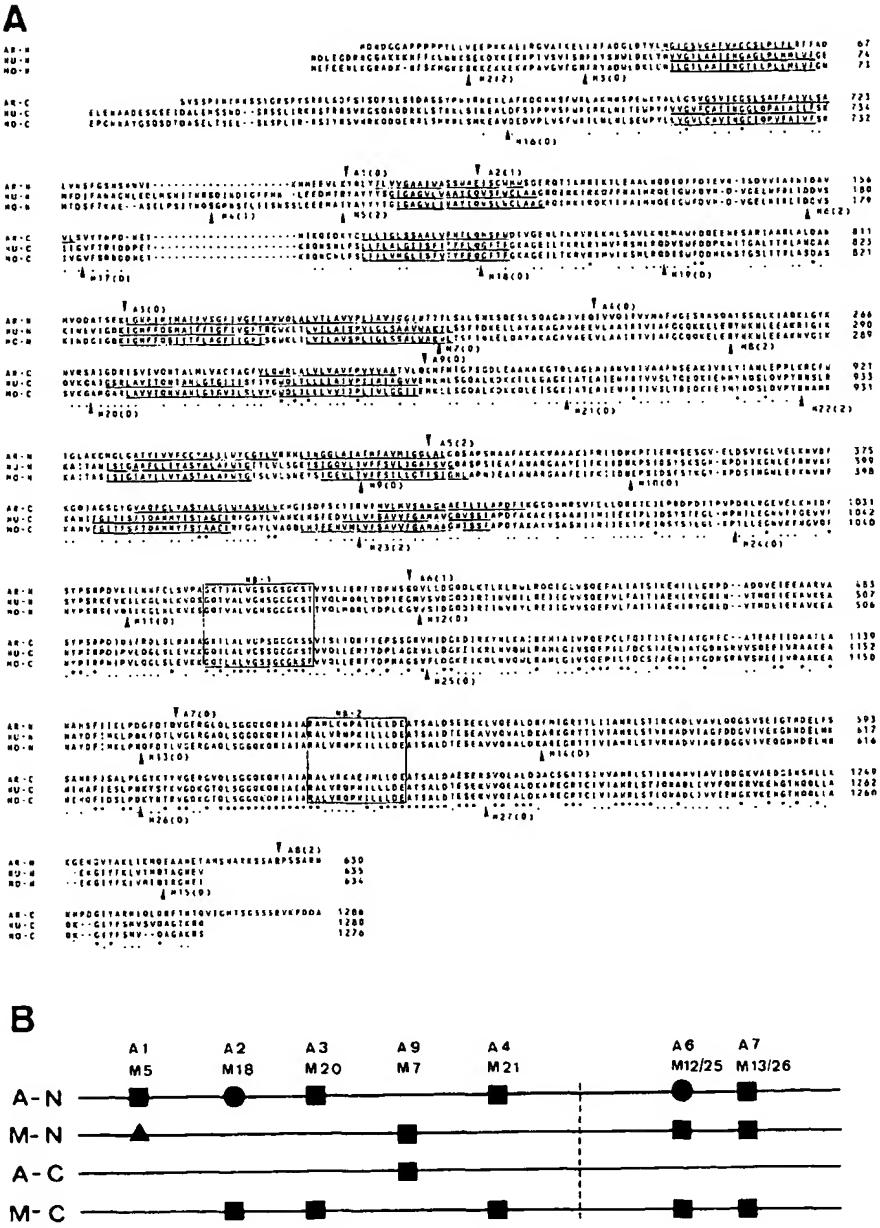

FIG. 6. *A*, alignment of putative *Arabidopsis atpgp1* gene product with mouse *mdr1* and human *MDR1* P-glycoproteins (Raymond and Gros, 1989; Chen *et al.*, 1990). The N-terminal halves of the *Arabidopsis atpgp1* product (*AR-N*), the human (*HU-N*), and the mouse (*MO-N*) P-glycoproteins were aligned with each other and the respective C-terminal halves (*AR-C*, *HU-C*, and *MO-C*) using the CLUSTAL program (PC/GENE software). *Dashes* mark gaps introduced to optimize the alignment. Amino acids that are identical or conserved in all six sequences are marked with *asterisks* and *periods*, respectively. *NB-1* and *NB-2* mark the conserved sequences (*boxed*) of the nucleotide-binding sites. Putative transmembrane domains are *underlined*. The positions of *Arabidopsis* introns (A1–A9) relative to the protein sequence are indicated by *arrowheads* pointing downwards above the sequences. Positions of the mammalian introns (M2–M27) are marked by *arrowheads* pointing upwards below the sequences. The *numbers* in *parentheses* indicate the intron type: type 0 introns interrupt the coding sequence between codons, types 1 and 2 introns interrupt within a codon after the first and second nucleotides, respectively. *B*, schematic positional map of relevant introns. The *lines* represent the N- and C-terminal halves of the *Arabidopsis* (A-N and A-C) and the mammalian P-glycoproteins (M-N and M-C). *Symbols* mark only the positions of introns that match approximately between the plant and the mammalian genes (labeled above the map). *Squares*, type 0; *circles*, type 1; *triangles*, type 2 introns. The map is not drawn to scale. The *stippled vertical line* represents the border between transmembrane domains (*left*) and nucleotide binding domains (*right*).

in different halves of the extant genes match, *i.e.* corresponding to the locations of A2/M18, A3/M20, A9/M7, A4/M21, M9/M23, A6/M12/M25, and A7/M13/M26 (Fig. 6). Following the duplication and subsequent fusion of the primordial gene, some introns wuld have been differentially lost from, and others gained by, the two halves during the separate evolution of animals and plants. The fact that the N-terminal half of the *Arabidopsis* P-glycoprotein exhibits a similar deletion (at amino acid 80; Fig. 6*A*), relative to the mammalian N-terminal halves, as both plant and mammalian C-terminal halves, is consistent with this interpretation.

Alternatively, for consistency with the hypothesis of Chen *et al.* (1990), one might postulate that the N-terminal half of the *Arabidopsis* P-glycoprotein is a homologue of the mammalian C-terminal halves and vice versa. Thus, *atpgp1* would represent a gene the two halves of which switched places relative to the mammalian *mdr* genes. This would explain the approximate match in the positions of intron pairs A2/M18, A3/M20, A4/M21, and A9/M7, but not of A1/M5. The match of the latter pair would then have to be attributed to coincidence. Interpreted in this way, all the arguments for the independent evolution of the two halves of the mammalian *mdr* genes proposed by Chen *et al.* (1990) could be applied to *atpgp1*. The fact that sequence comparisons revealed a closer relationship of the N- and C-terminal halves of the *Arabidopsis* protein to the corresponding halves of the mammalian P-glycoproteins would have to be explained by convergent evolution.

Considering these arguments, we think that intron positions and sequence relationships are more naturally explained by the first hypothesis than by the second and thus favor an evolutionary scenario in which P-glycoproteins evolved from the duplication and subsequent fusion of an intron containing primordial gene prior to the evolutionary separation of animals and plants.

Functional Considerations—MDR-like proteins form a

subfamily within the superfamily of ATP-binding cassette-containing (Hyde *et al.*, 1990) transport systems that share a conserved hydropathy profile and considerable sequence similarity mainly in the nucleotide-binding regions. Although not all members of this subfamily have been characterized functionally, it seems likely that they all are involved in some transport process. Best known are the mammalian P-glycoproteins, some of which were shown to be efflux pumps for a variety of large hydrophobic, but otherwise dissimilar drugs in multidrug-resistant tumor cells (see Endicott and Ling (1989) and Pastan and Gottesman (1991) for reviews). The normal function of these proteins is not known, but it has been hypothesized that it may be the elimination of toxic metabolic or xenobiotic substances. More distantly related but structurally similar mammalian proteins include the cystic fibrosis transmembrane conductance regulator (Riordan *et al.*, 1989) that has recently been shown to work as a regulated chloride channel (Anderson *et al.*, 1991; Rich *et al.*, 1991), the peroxisomal membrane protein PMP70 (Kamijo *et al.*, 1990) of undefined function, and the products of *mdr*-like genes located in the class II region of the major histocompatibility complex that may be involved in the transport of processed antigens to the surface of antigen-presenting cells (Deverson *et al.*, 1990; Trowsdale *et al.*, 1990; Spies *et al.*, 1990). The P-glycoprotein homologue described in the protozoan *P. falciparum* is involved in chloroquine resistance (Foote *et al.*, 1989), and a similar protein in *L. tarentolae* may be associated with methotrexate resistance (Ouellette *et al.*, 1990). In yeast, a P-glycoprotein homologue encoded by the *STE6* gene mediates the export of the proteinaceous a-factor mating pheromone (McGrath and Varshavsky, 1989). Prokaryotic members of the MDR-like subfamily include signal sequence independent protein export systems, *e.g.* for hemolysin and colicin V in *E. coli* (Felmlee *et al.*, 1985; Gilson *et al.*, 1990), as well as polysaccharide export systems (Stanfield *et al.*, 1988; Cangelosi *et al.*, 1989). In the face of the wide variety of known or inferred substrates of MDR-like proteins, a detailed discussion of possible substrates of the *atpgp1* gene product seems premature since it would remain purely speculative. However, the fact that the *Arabidopsis* protein exhibits the highest sequence similarity to the mammalian P-glycoproteins may suggest that functional aspects are also conserved. Thus, the *atpgp1* gene product may be involved in the extrusion of toxic metabolic and xenobiotic compounds from cells. It is interesting in this respect that the transcript from this gene is particularly abundant in peduncles. One may speculate that this reflects tissue-specific expression, possibly in vascular tissue. The cloned *atpgp1* gene now makes it possible to answer experimentally questions about its expression pattern and its function. For example, it will be interesting to test whether the *atpgp1* gene product can confer multidrug resistance to cells, and if so, to determine potential substrates, which may include herbicides.

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REFERENCES

Ames, G. F.-L. (1986) *Annu. Rev. Biochem.* **55**, 397–425

Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E., and Welsh, M. J. (1991) *Science* **253**, 202–205

Cangelosi, G. A., Martinetti, G., Leigh, J. A., Lee, C. C., Theines, C., and Nester, E. W. (1989) *J. Bacteriol.* **171**, 1609–1615

Cavener, D. R., and Ray, S. C. (1991) *Nucleic Acids Res.* **19**, 3185–3192

Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) *Cell* **47**, 381–389

Chen, C., Clark, D., Ueda, K., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1990) *J. Biol. Chem.* **265**, 506–514

Croop, J. M., Raymond, M., Haber, D., Devault, A., Arceci, R. J., Gros, P., and Housman, D. E. (1989) *Mol. Cell. Biol.* **9**, 1346–1350

Deverson, E. V., Gow, I. R., Coadwell, W. J., Monaco, J. J., Butcher, G. W., and Howard, J. C. (1990) *Nature* **348**, 738–741

Dudler, R., and Travers, A. A. (1984) *Cell* **38**, 391–398

Endicott, J. A., and Ling, V. (1989) *Annu. Rev. Biochem.* **58**, 137–171

Eisenberg, D., Schwartz, E., Komaromy, M., and Wall, R. (1984) *J. Mol. Biol.* **179**, 125–142

Felmlee, T., Pellett, S., and Welch, R. A. (1985) *J. Bacteriol.* **163**, 94–105

Foote, S. J., Thompson, J. K., Cowman, A. F., and Kemp, D. J. (1989) *Cell* **57**, 921–930

Gilson, L., Mahanty, H. K., and Kolter, R. (1990) *EMBO J.* **9**, 3875–3884

Gros, P., Croop, J., and Housman, D. (1986) *Cell* **47**, 371–380

Heap, J., and Knight, R. (1982) *Aust. J. Agric. Res.* **37**, 149–156

Higgins, C. F., Hiles, I. D., Salmond, G. P. C., Gill, D. R., Downie, J. A., Evans, I. J., Holland, I. B., Gray, L., Buckel, S. D., Bell, A. W., and Hermodson, M. A. (1986) *Nature* **323**, 448–450

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990) *Nature* **346**, 362–365

Kamijo, K., Taketani, S., Yokota, S., Osumi, T., and Hashimoto, T. (1990) *J. Biol. Chem.* **265**, 4534–4540

Kawasaki, E. S., and Wang, A. M. (1989) in *PCR Technology* (Erlich, H. A., ed) pp. 89–97, Stockton Press, New York

Lincke, C. R., Smit, J. J. M., Van der Velde-Koerts, T., and Borst, P. (1991) *J. Biol. Chem.* **266**, 5303–5310

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

McGrath, J. P., and Varshavsky, A. (1989) *Nature* **340**, 400–404

Moss, S. R., and Cussans, G. W. (1987) in *Biological and Chemical Approaches to Combating Resistances to Xenobiotics* (Ford, M., Holloman, D., Khambay, B., and Sawicki, R., eds) pp. 200–213, Society for Chemical Industry, London

Ng, W. F., Sarangi, F., Zastawny, R. L., Veinot-Drebot, L., and Ling, V. (1989) *Mol. Cell. Biol.* **9**, 1224–1232

Ouellette, M., Fase-Fowler, F., and Borst, P. (1990) *EMBO J.* **9**, 1027–1033

Pastan, I., and Gottesman, M. M. (1991) *Annu. Rev. Med.* **42**, 277–286

Putwain, P. D., and Collin, H. A. (1989) in *Herbicides in Plant Metabolism* (Dodge, A. D., ed) pp. 211–235, Cambridge University Press, Cambridge, United Kingdom

Raymond, M., and Gros, P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6488–6492

Rich D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E., and Welsh, M. J. (1991) *Science* **253**, 205–207

Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) *Science* **245**, 1066–1073

Samuelson, J., Ayala, P., Orozco, E., and Wirth, D. (1990) *Mol. Biochem. Parasitol.* **38**, 281–290

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467

Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars, R. (1990) *Nature* **348**, 744–747

Stanfield, S. W., Ielpi, L., O'Brochta, D., Helinski, D. R., and Ditta, G. S. (1988) *J. Bacteriol.* **170**, 3523–3530

Traut, T. W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2944–2948

Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1990) *Nature* **348**, 741–744

Van der Bliek, A. M., and Borst, P. (1989) *Adv. Cancer Res.* **52**, 165–203

Van der Bliek, A. M., Baas, F., Ten Houte de Lange, T., Kooiman, P. M., Van der Velde-Koerts, T., and Borst, P. (1987) *EMBO J.* **6**, 3325–3331

Wood, W. I., Gitschier, J., Lasky, L. A., and Lawn, R. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1585–1588

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Involvement of an ABC Transporter in a Developmental Pathway Regulating Hypocotyl Cell Elongation in the Light

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In the dark, plant seedlings follow the skotomorphogenetic developmental program, which results in hypocotyl cell elongation. When the seedlings are exposed to light, a switch to photomorphogenetic development occurs, and hypocotyl cell elongation is inhibited. We have manipulated the expression of the *AtPGP1* (for *Arabidopsis thaliana* P glycoprotein1) gene in transgenic Arabidopsis plants by using sense and antisense constructs. We show that within a certain light fluence rate window, overexpression of the *AtPGP1* gene under the control of the cauliflower mosaic virus 35S promoter causes plants to develop longer hypocotyls, whereas expression of the gene in antisense orientation results in hypocotyls shorter than those occurring in the wild type. In the dark, hypocotyls of transgenic and wild-type plants are indistinguishable. Because the *AtPGP1* gene encodes a member of the superfamily of ATP binding cassette–containing (ABC) transporters, these results imply that a transport process is involved in a hypocotyl cell elongation pathway active in the light. The AtPGP1 transporter is localized in the plasmalemma, as indicated by immunohistochemical techniques and biochemical membrane separation methods. Analysis of the *AtPGP1* expression pattern by using reporter gene constructs and in situ hybridization shows that in wild-type seedlings, *AtPGP1* is expressed in both the root and shoot apices.

INTRODUCTION

A young seedling's predominant endeavor is to reach favorable light conditions to switch from heterotrophic to photoautotrophic growth. Consequently, light, which serves as energy source, provides important information for plant morphogenesis. Plants make use of a sophisticated system of photoreceptors to sense the quality, quantity, periodicity, and direction of light (Kendrick and Kronenberg, 1994). At least three different photoreceptor systems are known to be involved in modulating development: phytochromes, which primarily absorb red and far-red light; blue light/UV-A receptors; and UV-B receptors (Chamovitz and Deng, 1995; Quail et al., 1995). Depending on the light conditions, seedlings can follow two developmental programs. In darkness, their hypocotyls elongate, the yellow cotyledons are kept closed, and apical hooks are formed (skotomorphogenesis). On the other hand, light triggers deetiolation, which results in inhibition of hypocotyl elongation, opening of apical hooks, unfolding of the cotyledons, and development of chloroplasts (photomorphogenesis). Recently, mutations at the *PROCUSTE1* locus that cause specific defects in hypocotyl cell elongation in dark-grown plants have been described in Arabidopsis (Desnos et al., 1996). The observation that light stimulates hypocotyl cell elongation in these mutant seedlings uncovered a novel *PROCUSTE1*-independent genetic pathway termed X that controls hypocotyl cell elongation in dim light (Desnos et al., 1996).

We previously cloned the multidrug resistance (*MDR*)-like gene *AtPGP1* (for *Arabidopsis thaliana* P glycoprotein1) from Arabidopsis (Dudler and Hertig, 1992). The products of *MDR*-like genes belong to the ubiquitously occurring family of ATP binding cassette–containing (ABC) transporters that mediate the ATP-driven transmembrane translocation of a large variety of substrates (Higgins, 1992; Gottesman et al., 1995). To analyze the function of *AtPGP1*, we manipulated its expression in transgenic plants. Here, we present evidence that AtPGP1 is involved in a hypocotyl cell elongation program that is active in dim light, thus implicating a transport function in this developmental process. Localization experiments and analysis of the *AtPGP1* expression pattern indicated that AtPGP1 is localized in the plasma membrane and that the corresponding gene is expressed in both the root and shoot apices of seedlings.

RESULTS

Generation and Analysis of Transgenic Arabidopsis Plants Constitutively Expressing *AtPGP1* Gene Constructs in Sense and Antisense Orientations

To analyze the function of the *AtPGP1* gene, we generated two series of transgenic Arabidopsis plants. Plants of the first series expressed the *AtPGP1* gene constitutively under the control of the cauliflower mosaic virus (CaMV) 35S

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promoter. In the second series, the suppression of AtPGP1 protein synthesis was attempted by constitutive expression of an antisense construct driven by the same promoter. The constructs were introduced into the Arabidopsis genome by using the Agrobacterium-mediated in-the-plant transformation system (Bechtold et al., 1993). Plants from the first six independently transformed lines homozygous for the 35S-*AtPGP1* gene (OE lines) and the first five lines homozygous for the antisense construct (AS lines) were analyzed in the T_3 generation. RNA gel blot analysis showed that in all OE lines, *AtPGP1*-specific mRNA of ~4.5 kb was much more abundant than in nontransformed wild-type plants (Figures 1A and 1B). In the AS lines, new RNA of the expected length of 1.9 kb as well as shorter fragments could be detected with a double-stranded *AtPGP1*-specific hybridization probe, whereas in most cases, the authentic mRNA of 4.5 kb was less abundant than in the wild type.

The transgene-mediated modification of *AtPGP1* expression could be confirmed at the protein level. Figures 1C and 1D show gel blots of microsomal membrane proteins probed with an AtPGP1-specific rabbit antiserum raised against a fusion protein produced in *Escherichia coli*. In extracts from OE plants, the antibody identified a single band of ~140 kD (Figure 1C). This is in good agreement with the theoretically calculated weight of 140.57 kD for AtPGP1. No signal was detected in the lane with proteins extracted from wild-type plants on this blot, which contained 10 μg of protein per lane, indicating that in wild-type plants, AtPGP1 is not an abundant protein. However, when 100 μg of protein was loaded per lane, the antibody detected two bands in extracts of wild-type plants in the size range of ~130 to 140 kD (Figure 1D).

In AS plants, both bands were visible as well, but in most cases, the upper one appeared to be reduced compared with the lower one (Figure 1D). To determine which of the two bands corresponded to AtPGP1, we co-electrophoresed membrane proteins from the AS2 line with an aliquot of OE3 protein (lane AS2/OE3 in Figure 1D). Because the upper band was enhanced by the addition of OE3 extract, it must represent AtPGP1, whereas the lower one most likely represents a cross-reacting homolog. The reduction of AtPGP1 in AS lines was quantified densitometrically by using the lower band as an internal standard. Figure 1E shows that the amount of AtPGP1 in the AS plants reached 30 to 80% of the wild-type level, depending on the individual transgenic line. These values may overestimate AtPGP1 levels in AS plants because it is possible that the upper band represents additional cross-reacting proteins. Thus, the antisense strategy to knock out *AtPGP1* expression had worked at least in part. For OE plants, the amount of AtPGP1 was estimated to be enhanced above that of the wild type by a factor of ~200.

AtPGP1 Mediates Hypocotyl Length in the Light

We observed that in comparison with the wild type, all six independently transformed OE lines used in this study exhibited elongated hypocotyls, whereas AS plants had shorter ones. In Figure 2, the average hypocotyl length of all lines is plotted from germination of the seeds until maximal elongation of the hypocotyl. Under the growth conditions used in this experiment (an 18-hr photoperiod with 75 μmol m^{-2} sec^{-1} white light at 24°C), hypocotyls of AS, wild-type, and

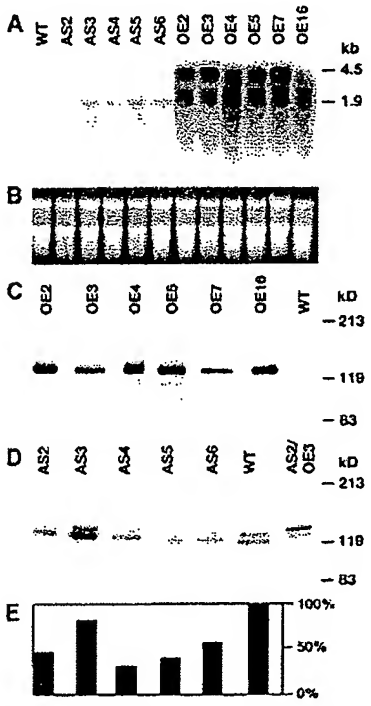

Figure 1. Analysis of *AtPGP1* Expression in Wild-Type and Transgenic AS and OE Plants.

(A) Autoradiography of an RNA gel blot probed with a double-stranded ^{32}P-labeled probe. Each lane contains 8 μg of total RNA. The expected lengths of *AtPGP1* and antisense transcripts are 4.5 and 1.9 kb, respectively. The lengths in kilobases of marker fragments are indicated at right. WT, wild type.

(B) Ethidium bromide–stained gel before blotting.

(C) and **(D)** Gel blot analysis of membrane proteins extracted from OE, AS, and wild-type plants. Ten and 100 μg of microsomal proteins were loaded per lane on the gels corresponding to the blots shown in **(C)** and **(D)**, respectively. The lane labeled AS2/OE3 in **(D)** contained 100 μg of AS2 and 2 μg of OE3 microsomal proteins. The blots were probed with an AtPGP1-specific rabbit antiserum and processed with the ECL chemiluminescent detection system. The sizes of the marker proteins are given at right in kilodaltons.

(E) Relative intensities of the upper band (corresponding to AtPGP1) to the lower band (serving as an internal standard) in corresponding lanes in **(D)**. The histogram represents the percentage values of this ratio relative to the wild type.

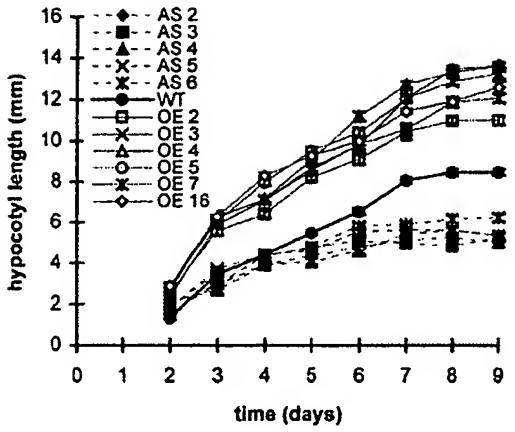

Figure 2. Analysis of the Hypocotyl Growth Rate of Wild-Type and Transgenic Plants.

The plants were grown under long-day conditions (18-hr photoperiod at 24°C) under white light at a fluence rate of 75 μmol m^{-2} sec^{-1}. Each data point represents the average length of 10 to 25 plants. For the wild type (WT), 20 to 35 hypocotyls were measured per day. The error bars indicate the standard error of the mean.

OE plants reached maximal average lengths of ~5, 8, and 12 mm, respectively, 9 days after germination.

The difference in hypocotyl length of wild-type, OE, and AS plants in theory may be caused by an increase in cell elongation, cell number, or both. To determine which parameter was affected by the transgene, we counted epidermal cells in the files in which they were arranged in the hypocotyl (Gendreau et al., 1997). As is evident from Figure 3A, AS plants had shorter and OE plants longer epidermal cells in their hypocotyl than did the wild type, whereas the number of cells in each file was identical in transgenic and wild-type plants (Figure 3B).

The phenotypic effect of the transgenes on the hypocotyl length could only be observed in plants that were grown in the light. The hypocotyl lengths of etiolated AS, OE, and wild-type seedlings exhibited no statistically significant difference. The data shown in Figure 4A originated from three different experiments. Seedlings were grown in a dark-growth cabinet and collected in single experiments after 5, 7, and 8 days to ensure that hypocotyls had reached maximal length. Hypocotyl lengths reached maximal values after 5 days and were not significantly different in transgenic and wild-type plants (Student's *t* test and ANOVA test; 95% confidence level).

Because light quality has a major impact on a young seedling's hypocotyl length (von Arnim and Deng, 1994), the response of the transgenic plants to far-red, red, and blue light with defined spectra was investigated. As shown in Figures 4B to 4D, the relative differences of hypocotyl lengths between the transgenic and wild-type plants remained qualitatively the same under all wavelengths. As previously described (Desnos et al., 1996), blue light inhibition of hypocotyl growth was stronger than the effect exerted by red light (Figures 4B and 4C). Interestingly, under red light, OE plants developed longer hypocotyls than they did in complete darkness (Figures 4A and 4B). Seedlings grown under red light showed the characteristic lack of negative gravitropism (Golan et al., 1996), indicating a bona fide red light response (data not shown). Under continuous far-red light, the seedlings remained very short (Figure 4D) and were deetiolated but never became green.

To determine the light dose response of transgenic and wild-type plants, we grew seedlings under different fluence rates of white light with a 6-hr night break, and maximal hypocotyl lengths were measured. As depicted in Figure 5, above a light fluence rate of ~0.5 μmol m^{-2} sec^{-1}, the hypocotyls of wild-type plants became longer with increasing

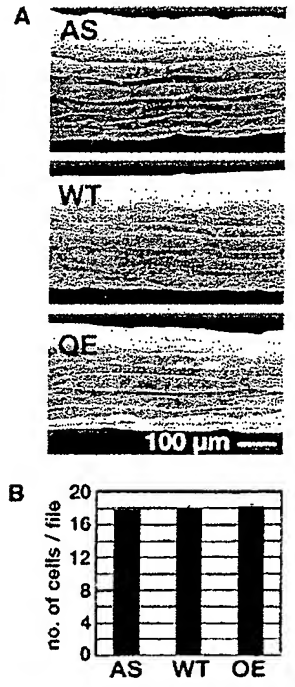

Figure 3. Number and Length of Hypocotyl Cells in 7-Day-Old Wild-Type, AS, and OE Plants Grown at a Light Fluence Rate of 52 μmol m^{-2} sec^{-1}.

(A) Scanning electron microscopy of hypocotyls.

(B) Number of epidermal cells per file in hypocotyls. Eight hypocotyls from each of two AS lines, two OE lines, and the wild type were scored. The error bars indicate the standard error of the mean. WT, wild type.

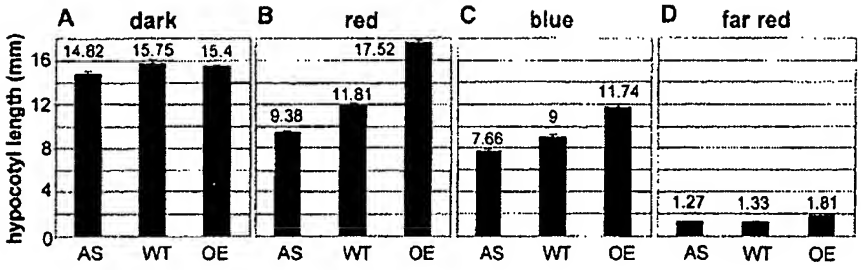

Figure 4. Influence of Light Quality on Hypocotyl Length in Wild-Type, AS, and OE Plants.

(A) Hypocotyl lengths of etiolated seedlings. The data represent the averages of three independent measurements conducted after 5, 7, and 8 days of growth in complete darkness. Hypocotyls already had reached maximal length after 5 days. A total of 142 AS and 190 OE seedlings of all transgenic lines and 102 wild-type seedlings were scored. The differences between OE, AS, and wild-type plants are statistically not significant at the 95% confidence level.

(B) to **(D)** Seedlings of two AS lines, two OE lines, and the wild type were grown in light of different qualities, and hypocotyls were measured at two different time points that yielded identical results, thus ensuring full elongation. In **(B)**, 40 seedlings per line were measured after 19 days of growth in red light at a fluence rate of 25 μmol m^{-2} sec^{-1}. In **(C)**, 20 seedlings per line were scored after 14 days in blue light at a fluence rate of 15 μmol m^{-2} sec^{-1}. In **(D)**, 18-day-old seedlings were grown under far-red light at a fluence rate of 2 μmol m^{-2} sec^{-1}. Twenty seedlings per line were scored.

Error bars indicate the standard error of the mean. WT, wild type.

light fluence rate and reached a maximal length at ~20 μmol m^{-2} sec^{-1}. Higher fluence rates resulted in shorter hypocotyls. The elongation of OE hypocotyls under dim light was greatly increased; in AS lines, it was essentially abolished. Hypocotyls in all lines decreased in length at higher light fluence rates and were indistinguishable at 100 μmol m^{-2} sec^{-1}, reaching a length of ~2 mm.

AtPGP1-Overexpressing Plants Have Longer Roots

As depicted in Figure 6, overexpression of *AtPGP1* also had a phenotypic effect on roots. Roots of OE plants grown on soil for 12 days under normal conditions (20 hr of light at a fluence rate of 75 μmol m^{-2} sec^{-1}) were slightly longer than the roots of wild-type and AS plants. The roots of the latter seemed to be shorter than those of the wild type, but in contrast to OE plants, the difference was not significant in statistical tests at a confidence level of 95%. Root length was also measured 8 days after germination with essentially the same results: OE roots were significantly longer than those of wild-type plants, whereas AS roots appeared to be slightly shorter, although the difference between AS and wild-type roots was statistically not significant (data not shown).

Apart from the hypocotyl and root phenotypes, no other phenotypic effect of the transgenes was observed in any OE and AS line throughout the complete life cycle of the plant. Because the products of *MDR*-like genes often are involved in resistance against toxic compounds and heavy metals (Ortiz et al., 1992; Gottesman et al., 1996), the transgenic plants also were tested for their tolerance to emetine and doxorubicine, two compounds known to induce multidrug resistance in mammalian cells, as well as for their resistance to the herbicides paraquat, azifluorfen, atrazine, and alachlor and the heavy metal cadmium sulfate. No difference between AS, wild-type, and OE plants was detected (data not shown).

AtPGP1 Expression Pattern of Wild-Type Plants

To examine the expression pattern of the *AtPGP1* gene, we placed the β-glucuronidase (GUS)-encoding *uidA* gene from *E. coli* under the control of the *AtPGP1* promoter (2.1 kb of 5' flanking sequence). After the introduction of this reporter gene into the Arabidopsis genome, kanamycin-resistant plants from 16 independent transgenic lines were analyzed in the T_1 generation by histochemical detection of GUS activity. In seedlings of all 16 independent lines, staining of the shoot apex was observed, whereas root apices exhibited GUS activity in 12 of the 16 lines (Figures 7A to 7D). In the shoot apex of these 16 lines, the meristematic region, leaf primordia, and young leaves as well as the proximal regions of the vascular tissue of cotyledons and the hypocotyl were stained, whereas the remaining parts of these organs never exhibited any GUS activity. Staining first became visible in the shoot apex ~24 hr after germination. As leaves expanded with progressive age, staining largely disappeared in these organs. Only the proximal part of the midrib frequently showed (12 of 16) weak GUS staining in fully expanded rosette leaves (data not shown). In 12 of the 16 lines, the elongation zone and frequently the specialization zone of roots, including the first root hairs, were stained (Figure 7D). The same pattern was observed in lateral roots, in which GUS activity

could be detected very early after initialization from cells in the pericycle.

Plants of 13 independent lines also were examined at flowering stages (data not shown). In all lines, the most intense GUS activity was associated with the nodes of the stem, including the bases of cauline leaves, the proximal part of the midrib, and the secondary inflorescence meristematic regions. In six of the 13 lines, the receptacles of flowers were stained, whereas other flower parts never exhibited GUS activity. In one of the six lines showing staining of the receptacles, the nectaries exhibited particularly strong GUS activity. Because this was observed in only one line, its significance is unknown. In roots, blue coloring was never observed at root tips, in contrast to the pattern in seedlings, but sometimes GUS staining was visible in the stele of relatively indistinct parts of the root system near bifurcations. It is not clear whether the weak staining in these older plant parts reflects activity of the reporter gene or just GUS activity that remained stable.

The GUS staining pattern was also observed in reporter plants of several lines subjected to all experimental conditions used in experiments with OE, AS, and wild-type plants. No change in the GUS staining pattern was observed in any of the experiments, indicating that *AtPGP1* expression is seemingly independent of changes in the environmental conditions.

The *AtPGP1* gene expression pattern was also analyzed by in situ hybridization of whole-mount preparations of roots and of longitudinal sections through the hypocotyl and shoot apices of Arabidopsis seedlings before germination and 12, 24, 72, 96, and 120 hr after germination. As is evident from examples shown in Figures 7E to 7N, the results

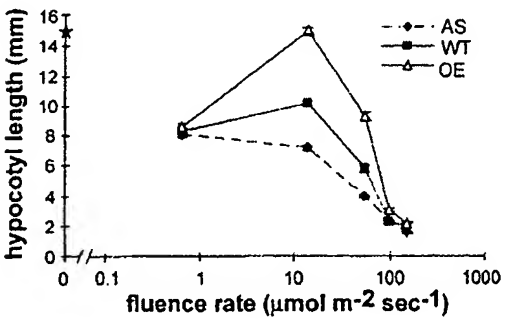

Figure 5. Influence of Light Quantity on Hypocotyl Length in Wild-Type, AS, and OE Plants.

Seedlings were grown under white light at different light fluence rates, and hypocotyls were measured at two different time points to ensure full elongation. The star on the vertical axis indicates the average hypocotyl length of etiolated seedlings (taken from the data presented in Figure 4). Data points represent measurements of 30 to 40 seedlings of the wild type (WT) and each of three AS and two OE lines, respectively. Error bars indicate the standard error of the mean.

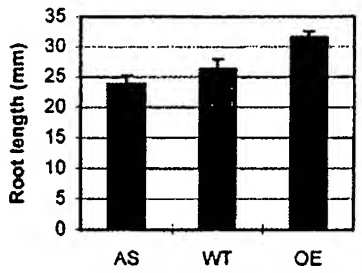

Figure 6. Root Length of Wild-Type, AS, and OE Seedlings.

Seedlings were grown on soil under white light at a fluence rate of 48 µmol m^{-2} sec^{-1}. Roots of two AS lines (30 seedlings), two OE lines (55 seedlings), and the wild type (WT; 19 seedlings) were scored after 12 days. Roots of OE plants were significantly different from those of AS and wild-type plants, whereas the difference between AS and wild-type roots was not statistically significant at the 95% confidence level. Error bars indicate the standard error of the mean.

of these experiments essentially confirmed the *AtPGP1* expression pattern inferred from the transgenic seedlings harboring the *GUS* reporter gene. Hybridization signals were not detected in longitudinal sections of vernalized seeds 12 hr after transfer to light and 24°C ambient temperature (Figures 7E and 7F) but appeared in the shoot apex and to a lesser extent in the cotyledons of seedlings 12 and 24 hr after germination, respectively (Figures 7G to 7J). Germination occurred 12 to 16 hr after transfer to normal growth conditions. In situ hybridization of longitudinal sections through hypocotyls of seedlings at any age never revealed a clear positive stain except for the region of the vascular strand most proximal to the apex (Figures 7K and 7L). The only slight divergence from the GUS staining pattern was found in roots, where in situ hybridization revealed a distinct signal at the region of the quiescent center (Figures 7M and 7N). In transgenic reporter plants, this region was not stained at all or was at the end of a decreasing gradient of GUS staining.

Subcellular Localization of AtPGP1

The subcellular localization of AtPGP1 as an integral membrane protein was examined by both immunohistochemical and biochemical techniques. Unfortunately, the polyclonal antibody raised against the AtPGP1 fusion protein was not suitable for immunohistochemical localization experiments because it unspecifically cross-reacted with tissue sections. Therefore, transgenic plants were generated harboring a modified CaMV 35S promoter-driven *AtPGP1* gene encoding a c-Myc epitope-tagged protein. The c-Myc epitope, which is recognized by the monoclonal antibody 9E10 (Evan et al., 1985), was introduced at position 1274 of the 1286-amino acid AtPGP1 protein. The construct was transferred

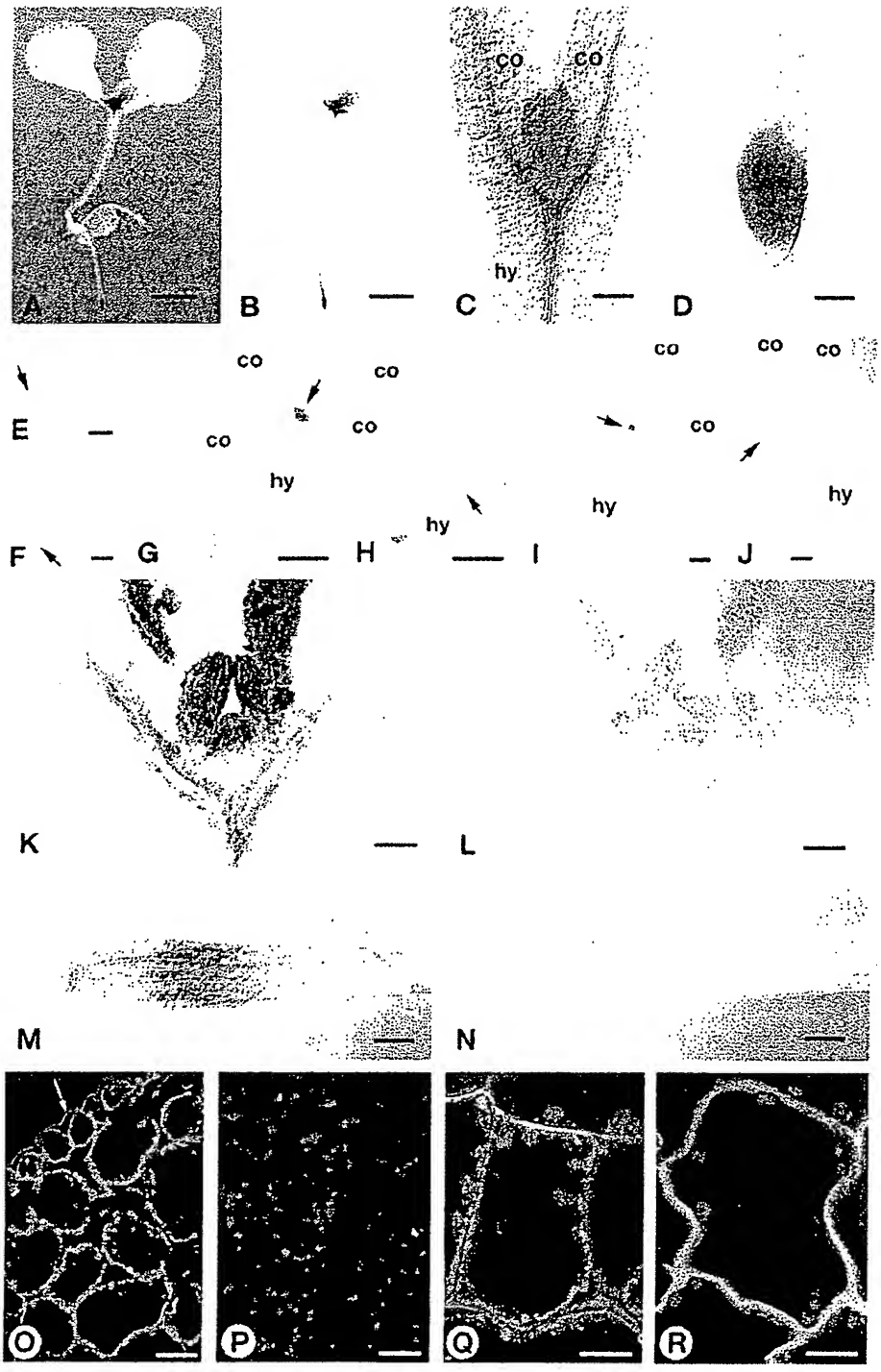

Figure 7. Localization of *AtPGP1* Gene Expression and of AtPGP1 Protein.

into the Arabidopsis genome by using the Agrobacterium-mediated transformation system. Hand-cut sections of homozygous transformants were fixed and probed with the anti-c-Myc antibody 9E10. To visualize bound antibody, we used a dichlorotriazynil aminofluorescein (DTAF)-conjugated secondary antibody.

Examination of immunostained leaf and meristem sections with a confocal laser scanning microscope revealed that the cells were exclusively labeled at the periphery, which is consistent with localization in the plasma membrane (Figures 7O and 7Q). Sections of wild-type control plants displayed no labeling, indicating that the signal very specifically represented the c-Myc epitope (Figures 7P and 7R). Experiments with transgenic plants expressing tagged AtPGP1 protein containing the c-Myc epitope at different positions (at Leu-14 near the N terminus and at Glu-80 in the first putative extracellular loop, respectively) revealed identical results (data not shown).

These results were confirmed by biochemical fractionation experiments. Microsomal membrane fractions were isolated from wild-type and transgenic plants expressing c-Myc epitope-tagged AtPGP1 and separated by continuous sucrose gradient centrifugation (Figures 8A and 8B). Gradient fractions were analyzed on protein gel blots. To determine the distribution of the different membrane compartments in the gradient, we used antibodies raised against VM23, a tonoplast protein (Maeshima, 1992); BiP, a protein localized in the lumen of the endoplasmic reticulum (Herman et al., 1994); and PIP, a plasmalemma protein (Kammerloher et al., 1994). As shown in Figure 8A, AtPGP1 from wild-type plants (detected with the AtPGP1-specific antiserum) and c-Myc epitope-tagged AtPGP1 from transgenic plants (detected with the 9E10 monoclonal antibody) migrated at the same positions in gradients run simultaneously. Both proteins comigrated with PIP, the plasmalemma marker protein, but not with the vacuolar VM23 and the endoplasmic reticulum-localized BiP proteins, indicating a location in the plasma membrane. These results are in good agreement with the findings of the immunocytochemical studies and show that despite the ectopic expression and the epitope-tagging of AtPGP1, the intracellular targeting was not affected.

DISCUSSION

Manipulation of AtPGP1 Levels in Transgenic Plants

To study the function of the *AtPGP1* gene, we constitutively and ectopically expressed it by using transgenic Arabidopsis plants in both sense and antisense orientations under the control of the CaMV 35S promoter. RNA and protein gel blot analyses showed that OE plants of the six independent lines analyzed contained much higher levels of both *AtPGP1*-specific RNA and protein than did wild-type plants. On protein gel blots, the antiserum raised against the AtPGP1 fusion protein recognized two bands of ~130 to 140 kD in membrane protein extracts of wild-type plants. We could show that the higher band at 140 kD corresponds to the AtPGP1 protein by superimposing AS and OE membrane protein samples. The AtPGP1 levels found in membrane preparations of the AS lines were estimated to range between 30 and 80% of the wild-type value. Although these values may underestimate the true reduction achieved because the 140-kD band may also represent cross-reacting AtPGP1 homologs, it is likely that some residual AtPGP1 is still present in all lines. The rough estimates of AtPGP1 levels that were obtained are not well correlated with the observed phenotype of individual AS lines. It is conceivable

Figure 7. (continued).

(A) to **(D)** Histochemical localization of GUS activity in transgenic plants harboring an *AtPGP1–GUS* reporter gene. In **(A)** and **(B)**, 7-day-old seedlings were photographed with two different background colors. In **(C)** and **(D)**, the shoot and root apex, respectively, of a 5-day-old seedling are shown. In **(A)** and **(B)**, bars = 1 mm; in **(C)** and **(D)**, bars = 100 μm.

(E) to **(N)** Detection of *AtPGP1*-specific RNA by in situ hybridization in wild-type seedlings. In **(E)** to **(L)**, longitudinal sections through seeds 12 hr after transfer from vernalization to growth conditions (**[E]** and **[F]**) and through seedlings 12 hr (**[G]** and **[H]**), 24 hr (**[I]** and **[J]**), and 5 days (**[K]** and **[L]**) after germination were hybridized with a digoxigenin-labeled *AtPGP1*-specific antisense RNA probe (**[E]**, **[G]**, and **[I]**) or with a sense RNA probe as a control (**[F]**, **[H]**, and **[J]**). In **(M)**, a whole mount of a root of a 5-day-old seedling was hybridized with a digoxigenin-labeled antisense RNA probe. In **(N)**, a section similar to the one shown in **(M)** was hybridized with a sense RNA probe as a control. Arrows indicate the shoot apex. Bars = 100 μm.

(O) to **(R)** Immunocytochemical localization of c-Myc epitope-tagged AtPGP1 in transgenic plants. False-color confocal laser scanning microscopy was performed with leaf sections probed with the c-Myc-specific monoclonal antibody 9E10. Detection was achieved with DTAF-conjugated secondary antibody (shown in green). The autofluorescence of chloroplasts is shown in red, whereas the purple color in **(Q)** and **(R)** indicates calcofluor white staining of cell walls. **(O)** and **(Q)** are transverse sections through cotyledon of a transgenic seedling carrying an ectopically expressed gene encoding c-Myc epitope-tagged AtPGP1. The arrow in **(O)** indicates the cuticle. **(P)** and **(R)** are control sections of wild-type seedlings treated exactly as the sections shown in **(O)** and **(Q)**. In **(O)** and **(P)**, bars = 20 μm; in **(Q)** and **(R)**, bars = 10 μm.

co, cotyledon; hy, hypocotyl.

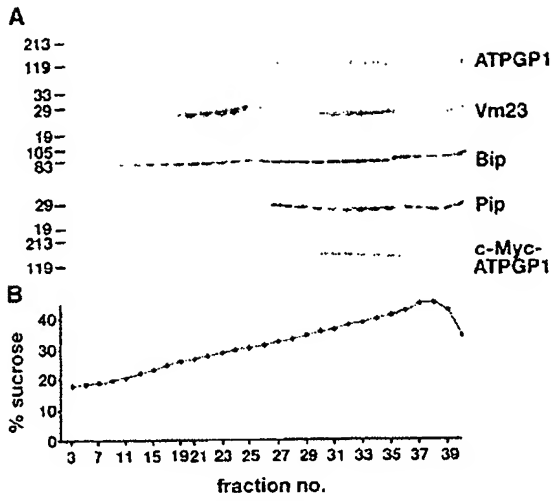

Figure 8. Protein Gel Blot Analysis of Membrane Vesicles Fractionated on Density Gradients.

(A) Membrane vesicles of wild-type Arabidopsis plants and transgenic plants ectopically expressing c-Myc epitope–tagged AtPGP1 were separated simultaneously on continuous sucrose density gradients. Gradients were fractionated, and appropriate aliquots of equal volume were separated on SDS–polyacrylamide gels and blotted. Shown are blots of wild-type samples of a single gradient probed with the AtPGP1-specific polyclonal antiserum or specific antisera to the tonoplast protein VM23, the endoplasmic reticulum protein BiP, and the plasmalemma protein PIP. The blot shown at bottom was prepared with samples derived from transgenic plants ectopically synthesizing c-Myc epitope–tagged AtPGP1 and was probed with the c-Myc epitope–specific monoclonal antibody 9E10. This gradient was run in conjunction with the one containing the wild-type samples, and the distribution of the marker proteins was identical in both gradients (data not shown). Numbers at left indicate the sizes in kilodaltons of marker proteins.

(B) Sucrose concentration of gradient fractions as measured with a refractometer.

that for wild-type function, a threshold level has to be present that was not reached in AS lines.

AtPGP1 Is Involved in a Hypocotyl Elongation Program Active in the Light

The skotomorphogenetic developmental pathway active in darkness is characterized by elongation of the hypocotyl and closed yellow cotyledons that form an apical hook. When exposed to light, seedlings switch from skotomorphogenetic to photomorphogenetic development, which results in inhibition of hypocotyl elongation, opening of the apical hook, unfolding of the cotyledons, and chloroplast development. Thus, light has an inhibitory effect on hypocotyl cell elongation.

Our experiments showed that within a certain light fluence-rate window, OE plants developed much longer hypocotyls and AS plants distinctly shorter ones than did wild-type plants. Outside of this fluence-rate window, plants of all three genotypes were indistinguishable. We conclude from these results that there exists a hypocotyl elongation program operating at relatively low-light fluence rates in which AtPGP1 is involved and that acts independently of the well-known light-induced hypocotyl elongation inhibition (Koornneef et al., 1980; Kendrick and Kronenberg, 1994). The observed hypocotyl length is the result of the superimposition of these two processes. Because of this superimposition, the light-stimulated hypocotyl elongation is relatively obscured in wild-type plants but is clearly detectable in comparison with AS plants. The fact that relative to the wild type, AS plants exhibited a hypocotyl phenotype opposite to the one observed in OE plants argues that AtPGP1 is involved in the light-stimulated hypocotyl elongation pathway in the wild type.

It turned out that light quantity rather than light quality had a distinct impact on the AtPGP1-mediated hypocotyl phenotype. Although seedlings exposed to continuous blue, red, and far-red light of defined spectra showed the characteristic appearance typical for the particular conditions, the phenotypic difference between the two classes of transgenic plants and the wild type remained qualitatively the same; therefore, it does not seem to depend on a particular range of wavelengths or a specific set of light receptors. This suggests that AtPGP1 acts downstream of a hypothetical point at which the signal transduction pathways of different photoreceptors converge. However, the experiment revealed an interesting result: under dim red light, AtPGP1-mediated hypocotyl elongation in OE seedlings more than compensated for the inhibition of hypocotyl elongation affected by the red light–induced deetiolation, resulting in longer hypocotyls than in etiolated seedlings (Figures 4A and 4B).

Elongation of the hypocotyl is known to be one component of the shade avoidance syndrome, which is induced by light with a low red-to-far-red ratio, because typically it is found under a canopy of dense vegetation (Yanovsky et al., 1995; Smith and Whitelam, 1997; Whitelam and Devlin, 1997). Because in our experiments neutral screens were used to dim white light, it can be ruled out that the observed hypocotyl elongation in the light is part of this syndrome. In addition, the effect would be expected to vanish in the red light experiments, in which this ratio is very high.

The existence of a genetic pathway that regulates Arabidopsis hypocotyl cell elongation in the light has recently also been proposed by Desnos et al. (1996), who described *procuste1* as being a mutant defective in hypocotyl cell elongation in the dark. Although *procuste1* seedlings had short hypocotyls in the dark, they were able to moderately elongate hypocotyls in dim light, thus uncovering a *PROCUSTE1*-independent light-stimulated hypocotyl elongation pathway

referred to as X by Desnos et al. (1996). The AtPGP1-mediated hypocotyl elongation pathway discovered in our experiments and the X pathway may well be identical, because both regulate hypocotyl cell elongation under nonsaturating light conditions in a dose-dependent manner. However, in contrast to Desnos et al. (1996), we observed a moderate increase of hypocotyl elongation with increasing light fluence rates under nonsaturating conditions in the wild type. This may be due to the different ecotypes used by the two groups (ecotype Columbia in the experiments of Desnos et al. [1996] and ecotype RLD in ours) or by differences in experimental conditions.

The Phenotype of Transgenic Plants and the Expression Pattern of *AtPGP1*

Reporter gene constructs and in situ hybridization experiments yielded a consistent *AtPGP1* expression pattern in seedlings. Strong GUS activity was found in the apical regions of both shoots and roots. The only difference was that in reporter plants, the root meristem either was not stained or was not distinctly stained, positioned at the end of a decreasing gradient of GUS activity, whereas in situ hybridization consistently revealed distinct signals in this region. This discrepancy could be explained by assuming lower GUS stability in the region of the quiescent center than in the surrounding tissue or, alternatively, by cross-hybridization of the probe with transcripts of a gene similar to *AtPGP1* that is expressed in this region.

The most surprising fact about the *AtPGP1* expression pattern is that neither GUS activity nor in situ hybridization signals were clearly detected in the hypocotyl itself, indicating that *AtPGP1* may not be expressed in the organ that is affected by antisense-mediated suppression of *AtPGP1* expression (see below). The sections of seedlings 12 hr after germination (shown in Figures 7G and 7H) have been deliberately overstained, resulting in a weak signal in the hypocotyl of the control section hybridized with the sense probe (Figure 7H). The corresponding signal on the section hybridized to the antisense probe is not significantly stronger, in contrast to the signals over the cotyledons. In summary, we were unable to unequivocally detect *AtPGP1* expression in the hypocotyl at any time point during and after germination. However, this does not exclude the possibility that *AtPGP1* gene product is present in hypocotyl cells either as the result of low-level expression during or after germination or as a result of expression during embryogenesis. The *AtPGP1* expression pattern in seedlings, as revealed by reporter plant examination and RNA gel blots, was not detectably altered by different light and other environmental conditions.

The observation that OE plants have longer roots than do wild-type plants is intriguing, considering *AtPGP1* expression in the wild-type root tip. However, because AS plants did not have significantly shorter roots, it is unclear whether the *AtPGP1* gene has a role in a root length regulation pathway in the wild type. The root phenotype in OE plants may be due to the ectopic nature of *AtPGP1* expression rather than to enhanced expression in root tips. On the other hand, it is possible that AS plants exhibit a root phenotype under other experimental conditions.

After bolting, high GUS activity was associated with the axillary meristematic regions and adjacent tissue, including the phloem and parenchyma in the basal regions of stem leaves. In flowers, the receptacle was the only part that in some lines (six of 13) showed GUS activity. The activity persisted until silique development was completed. This late activity may reflect stability of the enzyme rather than persisting synthesis. No phenotypic effect of the manipulation of *AtPGP1* expression was discovered in transgenic plants after the seedling stage.

How May AtPGP1 Affect Hypocotyl Cell Length?

Hypocotyl cells respond directly or indirectly to diverse environmental stimuli such as wavelength composition, fluence rate, and direction of the incident light. In some cases, perception and signaling may be cell autonomous (Neuhaus et al., 1993); otherwise, sites of perception and of action are apart in separate cells. Therefore, signals have to emanate from the site of perception to regulate directly or indirectly the length of the hypocotyl cells appropriately. An attractive hypothesis is that the AtPGP1 protein is involved in the transport of such a signal. A conceivable function of AtPGP1 would therefore be the export of a hormone-like compound from the shoot apical region that would regulate hypocotyl cell length. The production of this compound would be under the control of the light fluence rate perceived by the cotyledons and possibly other, yet unknown factors, whereas its export would be mediated by the constitutive AtPGP1 exporter. If the distribution of AtPGP1 is indeed confined to the apical regions, as suggested by our data, then the AtPGP1-exported signal would have to reach hypocotyl cells by diffusion into the apoplast or by transport through the vascular strand to induce cell expansion in competent cells. This signal is unlikely to be one of the basic plant hormones, because if it were, one would expect that constitutive ectopic expression of *AtPGP1* would have greater phenotypic consequences than those that have been observed. Alternatively, if AtPGP1 transporter is present in the plasmalemma of hypocotyl cells, it may be involved in the import of an incoming signal molecule, although, with the exception of the bacterial periplasmic binding protein–dependent importers, all ABC transporters analyzed have been reported to function in an export process (Higgins, 1992).

One conceivable hypothesis is that the substrate of the AtPGP1 exporter is a peptide hormone. MDR-like transporters have been shown to provide an export mechanism for peptides that is independent of the classic N-terminal transit sequence, as, for example, in yeast, in which the STE6 transporter is involved in the secretion of the 12-amino-acid-long

a-mating-type pheromone (Kuchler et al., 1989; McGrath and Varshavsky, 1989), or in mammals, in which the MDR-like TAP1/TAP2 complex transports processed antigens into the endoplasmic reticulum for assembly with major histocompatibility complex class I proteins (Williams et al., 1996). In plants, two prominent hormone-like peptides, systemin and early nodulin ENOD40, have been described (McGurl et al., 1992; van de Sande et al., 1996). Both lack a hydrophobic leader peptide in the active form as well as during their synthesis, but they are still exported out of cells, conceivably by ABC transporters.

There is increasing evidence that a variety of cellular processes, among them defense responses, growth, and development, are mediated by small peptides serving as signaling molecules (Pearce et al., 1991; McGurl et al., 1992; Matsubayashi and Sakagami, 1996; van de Sande et al., 1996; John et al., 1997; Miklashevichs et al., 1997). Although one can only speculate on the mechanism mediating hypocotyl cell elongation in the light at present, our results implicate a transmembrane transport function in this regulatory pathway. The identification of the substrate that is translocated by AtPGP1 will help us to understand this pathway, and this task should be facilitated by the transgenic plants described.

METHODS

Plant Growth Conditions

Arabidopsis thaliana ecotype RLD (stock No. N913; Arabidopsis Stock Centre, Nottingham, UK) was grown on Florabella cactus soil (Klasmann/Deilmann, Geeste, Germany) in a growth chamber (18 hr of light and 6 hr of darkness at 24°C) after a 4-day vernalization period (4°C in dim light) by using Arasystem containers (Lehle Seeds, Round Rock, TX). For growth under sterile conditions, seeds were surface sterilized (5 min in 70% ethanol, followed by a 15-min incubation in 5% [v/v] sodium hypochlorite, 0.2% [v/v] Tween 20, and a threefold rinse in sterile distilled water) and sown on half-strength Murashige and Skoog (MS) salts (Sigma) supplemented with 1% sucrose, 1 × Gamborg's vitamins, 0.5 g/L Mes, pH 5.7, and 0.8% (w/v) phytagar (Gibco BRL) in Petri dishes.

Light Conditions

White Light

Biolux fluorescent tubes (Osram, Munich, Germany) were used as sources for white light.

Red Light

The light of seven white fluorescence tubes (warm white, model L20 W/30 S; Osram) was filtered through 3 mm of red Plexiglas (red No. 501; Röhm and Haas, Darmstadt, Germany), resulting in a spectrum with a peak at 610 nm and a half bandwidth of ~60 nm.

Blue Light

The light of seven blue fluorescent tubes (No. 8531; Philips, Eindhoven, The Netherlands) was filtered through a Plexiglas screen (blue No. 627; Röhm and Haas) to give a peak at 455 nm with a half bandwidth of 30 nm.

Far-Red Light

The light from 10 light bulbs (Centra; Osram) was filtered through a combination of red (3 mm, No. 501) and blue (6 mm, No. 627) Plexiglas to obtain a far-red spectrum with a cutoff below 710 nm, reaching a maximum at 750 nm. Cheesecloth and a white mesh were used as neutral screens to dim the light. The individual fluence rates were obtained by moving the plants vertically closer to or away from the light source. The light fluence-rate was measured with a quantum sensor (model Li-190SB; Li-Cor, Lincoln, NE). All wavelengths and spectra were controlled with a Li-Cor spectroradiometer (model Li-1800).

Test for Resistance to Toxic Agents

Plants were grown for up to 3 weeks in liquid MS medium supplemented with test agents on a rotary shaker at 90 rpm. The herbicides atrazine (Novartis AG, Basel, Switzerland), azifluorfen (Rhône-Poulenc Rorer GmbH, Cologne, Germany), and paraquat (Chevron Chemical Co., San Francisco, CA) were tested in a range of 0.01 to 0.05 μg/mL; alachlor (Monsanto, St. Louis, MO) was tested in a range of 0.05 to 0.5 μg/mL. The drugs doxorubicine (Sigma) and emetine (Sigma) were applied at a concentration range of 0.1 to 100 μg/mL.

Measurement of Hypocotyl and Root Length

Seedlings were fixed in 70% ethanol and spread on agar plates. The plates were scanned on a flatbed scanner, and hypocotyl lengths were determined on the digitized image with the help of a standard using the National Institutes of Health (Bethesda, MD) Image software package (Macintosh version 1.60). To determine the root length of soil-grown plantlets, we removed the pot and suspended the soil containing the plantlets in water to clean the seedlings. Seedlings with intact roots were processed as described above.

Determination of Hypocotyl Cell Numbers and Scanning Electron Microscopy

Seedlings were cleared by incubation for 24 hr in a chloral hydrate–H_2O mixture (2.5:1), and the number of cells per file was determined under a microscope. For scanning electron microscopy, seedlings were fixed for 24 hr in a solution containing 4% formaldehyde, 50% acetone, and 5% acetic acid. After dehydration in 100% acetone, critical point drying, and coating with gold, they were analyzed with a scanning electron microscope (model S-4100; Hitachi, Yokohama, Japan).

Construction of Transformation Plasmids

GUS *Reporter Construct*

The starting plasmid was a 4-kb PstI fragment in pBluescript SK+ (Stratagene, La Jolla, CA) containing the 5′ part of the *AtPGP1* gene, including ~2.1 kb of the 5′ flanking region (Dudler and Hertig, 1992). From this plasmid, a 2.1-kb promoter fragment was cut out with HindIII (which cleaves in the multiple cloning site on the upstream side) and BclI (cutting in the leader region ~12 bp downstream of the transcriptional initiation site in the *AtPGP1* gene [nucleotide position 804; Dudler and Hertig, 1992]) and cloned between the HindIII and BamHI sites of the multiple cloning site of the plant transformation vector pBI101 (Clontech, Palo Alto, CA) upstream of the *GUS* gene.

Overexpression Construct

The starting plasmid was the one used for the *GUS* reporter construct. The 2.1-kb *AtPGP1* promoter fragment was cut out with AccI. AccI cuts in the multiple cloning site on the upstream side of the 4-kb PstI fragment and in the leader region ~25 bp downstream of the transcriptional initiation site in the *AtPGP1* gene (nucleotide position 817; Dudler and Hertig, 1992) and was replaced with a synthetic double-stranded oligonucleotide containing a BamHI restriction site (upper strand, 5′-CGTGGGATCC; lower strand, 5′-CTGGATCCCA). The upstream PstI site in the polylinker was lost during this manipulation. Thus, the 5-kb PstI fragment containing the rest of the *AtPGP1* gene and 3′ flanking sequences (Dudler and Hertig, 1992) could be cloned into the remaining downstream PstI site of this plasmid.

One of the resulting plasmids with the correct orientation of the 5-kb PstI fragment was selected and named pOE. The complete but promoterless 7-kb *AtPGP1* gene was cut out from pOE with BamHI, which cleaves in the newly introduced oligonucleotide and in the multiple cloning site downstream of the insert. The 7-kb BamHI fragment was inserted into the BamHI cloning site of the plant transformation vector pBI121 (Clontech), from which the *GUS* gene had been deleted previously by digestion with SmaI and SacI, with religation of the vector fragment after blunting the SacI end with T4 DNA polymerase. One of the resulting plasmids with the correct orientation of the *AtPGP1* gene relative to the cauliflower mosaic virus (CaMV) 35S promoter of the modified pBI121 vector was selected for plant transformation and named pBIMDR1.

Antisense Construct

The antisense construct contained the first 1.9 kb from the 5′ end of an *AtPGP1* cDNA in an antisense orientation between the CaMV 35S promoter and the nopaline synthase gene termination sequences of pBI121. The cDNA fragment was obtained using the reverse transcriptase–polymerase chain reaction procedure, as described by Dudler and Hertig (1992). Briefly, the 1.9-kb cDNA was amplified in three slightly overlapping pieces by using the first three primer pairs, as indicated in Figure 2 of Dudler and Hertig (1992). All primers had additional nucleotides at the 5′ ends to give suitable restriction sites for cloning of the resulting fragments. Thus, the most upstream piece of ~320 bp was flanked by SacI (5′) and XbaI (3′) sites; the slightly overlapping middle piece of ~680 bp had a SacI (5′) site and an XbaI (3′) site; and the most downstream piece of ~890 bp, which slightly overlapped with the middle fragment, was flanked by XbaI (5′) and BamHI (3′) sites.

These fragments were each cloned into pBluescript SK+ to yield pMDRPCR1 (upstream fragment), pMDRPCR2 (middle fragment), and pMDRPCR3 (downstream fragment). pMDRPCR1 and pMDRPCR2 contained a unique NcoI site in the part at which they overlapped (nucleotide position 1097; Dudler and Hertig, 1992). Thus, the short NcoI-XbaI fragment of pMDRPCR1 was replaced with the NcoI-XbaI fragment of pMDRPCR2 to give pMDRPCR12. The XbaI-BamHI insert of pMDRPCR3 was then cloned into pMDRPCR12. Because the XbaI sites of the two plasmids were at identical positions relative to the gene sequence (which contains five of the six nucleotides of the recognition sequence), the resulting plasmid (pMDRPCR123) contained 1.9 kb of cDNA sequences identical to the exon sequences of *AtPGP1*, except for the one-nucleotide change (T instead of a G residue at position 2037; Dudler and Hertig, 1992) that resulted in the XbaI site. The 1.9-kb insert of pMDRPCR123 was cut out with SacI and BamHI and used to replace the *GUS* gene of pBI121, resulting in pBIAS1.9.

c-Myc AtPGP1 Construct

A synthetic double-stranded oligonucleotide with AatII-compatible sticky ends (top strand, 5′-AGAGCAGAAGCTTATCTCCGAGGAGGACCTTACGT; bottom strand, 5′-AAGGTCCTCCTCGGAGATAAGCTTCTGCTCTAACGT) encoding the c-Myc epitope EQKLISEEDL (Evan et al., 1985) was designed for ligation into the AatII site of pOE (see above) at nucleotide position 5789 of the *AtPGP1* gene (Dudler and Hertig, 1992). The c-Myc epitope is thereby inserted at amino acid 1274 of the 1286–amino acid protein. The resulting clones were sequenced, and one with the insert in the correct orientation was selected and named pOEmyc. The modified *AtPGP1* gene was cut out from pOEmyc with BamHI and inserted into the BamHI cloning site of the plant transformation vector pBI121 from which the *GUS* gene had been previously deleted, as described above. One of the resulting plasmids with the correct orientation of the insert relative to the CaMV 35S promoter was selected for plant transformation and named pBIMDRMyc1.

Generation of AtPGP1-Specific Antiserum

A 613-bp BglII-HindIII fragment derived from pMDRPCR3 (see *Antisense Construct*, above) was cloned into the corresponding restriction sites of the bacterial expression vector p6xHis-DHFRS(−2) (Stüber et al., 1990). The resulting plasmid permitted isopropyl β-D-thiogalactopyranoside–inducible expression in *Escherichia coli* of a fusion protein consisting of an N-terminal histidine hexamer followed by a derivative of the mouse dihydrofolate reductase fused to part of the AtPGP1 protein (amino acids 340 to 544) that contained the first ATP binding domain. The fusion protein was purified from *E. coli* lysates by nickel chelate affinity chromatography using its histidine affinity tag, according to Stüber et al. (1990). The eluted protein was further subjected to SDS-PAGE. After Coomassie Brilliant Blue R 250 staining, the gel slice containing the fusion protein was cut out, crushed, and directly used to immunize rabbits. Immunization and preparation of antiserum were performed by Eurogentec (Seraing, Belgium).

Plant Transformation and Selection

Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 (Van Larebeke et al., 1974) as described

by Wen-jun and Forde (1989). The in-the-plant Agrobacterium-mediated transformation by vacuum infiltration was used as described previously (Bechtold et al., 1993), with the following modifications. Plants were infiltrated for 20 min without using a vacuum, and the infiltration medium (2.3 g/L MS salts, 0.112 g/L Gamborg's B5 vitamins, 0.5 g/L Mes, 5% [w/v] sucrose, and 0.044 μM 6-benzylaminopurine) contained 0.03% Silwet L-77 (Union Carbide Corp., Danbury, CT). T_1 seeds were collected, dried at 29°C, and sown on sterile media containing 50 μg/mL kanamycin to select the transformants. Surviving T_1 plantlets were transferred to soil to set seeds (T_2). The segregation frequency of the T_2 generation with regard to kanamycin resistance was determined on selective media. Seeds (T_3) of transgenic lines segregating for kanamycin resistance in a Mendelian ratio of 3:1 typical for a single integration locus were collected and again sown on selective media. T_2 plants were assumed to be homozygous for the transgene if all of 50 to 150 progeny seedlings were kanamycin resistant.

RNA Extraction and Gel Blot Analysis

After homogenizing plant tissue in liquid nitrogen with a mortar and pestle, we prepared total RNA by the hot phenol method and LiCl precipitation, as described previously (Dudler and Hertig, 1992). RNA gel blot preparation and hybridization were performed according to standard procedures (Maniatis et al., 1982). The ^{32}P-labeled 1.9-kb insert of pMDRPCR123 was used as a probe.

Protein Extraction and Gel Blot Analysis

Arabidopsis tissue was homogenized in 3 volumes of homogenization buffer (50 mM Hepes-KOH, pH 7.5, 5 mM EDTA, 0.1% BSA, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, 1% [w/v] polyvinylpolypyrrolidone, 0.1 mg/mL butylated hydroxytoluene, and 0.25 M sucrose) in a Waring blender, filtered through two layers of cheesecloth, and centrifuged at 7000*g* for 15 min to remove debris. The microsomal membrane fraction was pelleted from the supernatant by ultracentrifugation at 100,000*g* for 30 min. The pellet was resuspended in a buffer containing 5 mM Tris-HCl, pH 7, 2 mM EDTA, and 250 mM sucrose with the aid of a Potter homogenizer (Huber, Reinach, Switzerland). Protein concentrations were determined using the Bio-Rad protein assay. Aliquots were denatured by the addition of a 0.25 volume of 5 × sample buffer (60 mM Tris-HCl, pH 6.8, 15% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue), incubation at 60°C for 15 min, and sonication for another 15 min in a sonication water bath (47 kHz; Bransonic, Aasoest, The Netherlands). It was of crucial importance that the membrane vesicles were denatured in the largest possible volume; otherwise, AtPGP1 did not enter the separating gel.

Proteins were separated on 7% SDS–polyacrylamide gels and transferred to nitrocellulose filters (0.45-μm pore size; Millipore, Bedford, MA) by using a semidry blotting apparatus (Pharmacia-LKB, Uppsala, Sweden). For immunodetection of AtPGP1, the blots were blocked in TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% [v/v] Tween 20) containing 7.5% (w/v) nonfat dry milk powder and incubated in a 1:1000 dilution of anti-AtPGP1 antiserum. Blots were washed, incubated with goat anti-rabbit horseradish peroxidase–conjugated secondary antibody, and developed using a chemiluminescent immunodetection system (ECL; Amersham International) according to the manufacturer's directions. Milk powder was included in all incubation steps and washes except the last wash before the addition of the detection substrate.

For densitometric analysis, the autoradiogram of the gel blot was scanned with a flatbed scanner, and the resulting image was processed with the National Institutes of Health Image software package (Macintosh version 1.60), which allowed integration and quantification of signals.

Density Gradient Fractionation of Membrane Vesicles

The microsomal membrane fraction was isolated as described above, but the vesicles were resuspended in gradient buffer (10 mM Tris-Mes, pH 7.0, 1 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride) containing 250 mM sucrose. A continuous gradient from 15 to 45% (w/v) sucrose in gradient buffer was poured with the aid of a gradient former (Auto Density Flow II; Buchler Instruments, Fort Lee, NJ). After ultracentrifugation at 49,000 rpm for 6 hr in a TV 850 rotor (Sorvall, Newtown, CT), gradient fractions were collected, and 0.2 mL of 5 × sample buffer was added to each 0.8-mL fraction. Fractions were frozen in liquid nitrogen and stored at −80°C until used. Protein gel electrophoresis, blotting, and immunodetection of AtPGP1 were performed as described above. To detect c-Myc–tagged AtPGP1, monoclonal antibody 9E10 (1:1000; Calbiochem, La Jolla, CA) and horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:3000; Bio-Rad) were used. The antibodies against marker proteins were a gift of A.R. Schäffner, Ludwid-Maximilian University, Munich, Germany (chicken anti-PIP; Kammerloher et al., 1994), M. Chrispeels, University of California, San Diego (rabbit anti-BiP), and M. Maeshima, Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan (rabbit anti-VM23; Maeshima, 1992). The antisera were used at a dilution of 1:1000, and detection was achieved with a 1:3000 dilution of the corresponding specific horseradish peroxidase–coupled secondary antibody (Bio-Rad) and the ECL chemiluminescent detection system (Amersham International).

Histochemical GUS Staining

Histochemical detection of GUS activity was performed with whole seedlings or plant parts. As negative controls, untransformed plants were used. Tissue was vacuum infiltrated for 2 min with fixing solution (0.01% Silwet L77 detergent [Union Carbide Corp.], 0.3% formaldehyde, 10 mM Mes, pH 6.5, and 0.3 M mannitol) and incubated for 45 min at room temperature. After washing three times in 50 mM $NaPO_4$, pH 7, the tissue was incubated in the same buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid at 37°C for 16 hr. Chlorophyll was then eluted by incubation in 70% ethanol at 37°C.

In Situ Hybridization

Digoxigenin-labeled sense and antisense RNA probes were synthesized using the digoxigenin RNA labeling kit (Boehringer Mannheim). The template was pMDRPCR4, a pBluescript SK+ vector containing a 1080-bp-long partial *AtPGP1* cDNA amplified by a reverse transcriptase-polymerase chain reaction procedure that covered part of exon 8, exon 9, and part of exon 10 (from positions 3418 to 4657 in Figure 1 of Dudler and Hertig [1992]). In situ hybridization to 8-μm-thick Paraplast (Sigma) sections of 3- to 5-day-old formaldehyde-fixed Arabidopsis seedlings was performed according to Jackson

(1991), with the modifications described by Lincoln et al. (1994). Riboprobes were used at a concentration of ~0.6 ng/μL. Whole-mount in situ hybridization was performed with roots of 3- to 5-day-old seedlings of Arabidopsis according to Tautz and Pfeifle (1989) and exactly as described by Ludevid et al. (1992).

Tissue Sections and Immunohistochemistry

Seeds were germinated and grown submerged in liquid MS medium at 24°C on a rotary shaker at 90 rpm for 10 to 14 days. Leaves and stems were placed on a slice of polystyrol, and sections were cut by hand as thin as possible with a razor blade. Sections were immediately transferred to microtiter plates and fixed with pFA fixative (3.5% *p*-formaldehyde, 50 mM sucrose, 0.01% Silwet L-77, and 90 mM sodium phosphate, pH 6.8) for at least 3 hr at room temperature. Sections were washed for 30 min and then twice for 10 min in washing buffer (90 mM sodium phosphate and 0.2% glycine, pH 7.2), incubated in blocking buffer (150 mM NaCl, 1.5% [w/v] BSA, 0.03% [w/v] NaN_3, and 50 mM sodium phosphate, pH 7.5) overnight at 4°C, and finally incubated with a 1:3000 dilution of the monoclonal antibody 9E10 (Calbiochem) in blocking buffer for 24 hr at 4°C. The tissue sections were then washed twice for 15 min with blocking buffer and incubated in a 1:150 dilution of dichlorotriazynil aminofluorescein (DTAF)–coupled goat anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA) in blocking buffer. All following steps were conducted under light-tight conditions. After an incubation period of 18 to 24 hr at 4°C, sections were washed twice with blocking buffer and once with 90 mM sodium phosphate, pH 7.5. For nuclear staining, sections were incubated in 3 μg/mL 4′,6-diamidino-2-phenylindole (Sigma) in 90 mM sodium phosphate, pH 7.5, for 45 min. Cell walls were stained with 0.02% (w/v) calcofluor white M2R (Sigma) in 90 mM sodium phosphate, pH 7.5, for 5 min. Finally, the sections were rinsed once in 90 mM sodium phosphate, pH 7.5, and incubated for 5 min in mounting solution (50% glycerol, 0.02% *p*-phenylenediamine, 75 mM NaCl, and 50 mM Tris-HCl, pH 8) before being mounted on a microscope slide and covered with a coverslip, the edges of which were sealed with nail polish.

The sections were examined with an inverted confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with the appropriate filter sets (BP 522/32-FITC [Leica] for DTAF, LP 590 nm [Leica] for the autofluorescence of chloroplasts, and BP 455/30-DAPI [Leica] for DAPI and calcofluor white M2R). Fluorescence images of the separate channels were digitized with the Leica Scanware software and merged by using Adobe Photoshop version 4.0 (Adobe Systems Inc., San Jose, CA).

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REFERENCES

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Ser. III Sci. Vie **316,** 1194–1199.

Chamovitz, D.A., and Deng, X.W. (1995). The novel components of the Arabidopsis light signaling pathway may define a group of general developmental regulators shared by both animal and plant kingdoms. Cell **82,** 353–354.

Desnos, T., Orbovic, V., Bellini, C., Kronenberger, J., Caboche, M., Traas, J., and Höfte, H. (1996). *procuste1* mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. Development **122,** 683–693.

Dudler, R., and Hertig, C. (1992). Structure of an *mdr*-like gene from *Arabidopsis thaliana*: Evolutionary implications. J. Biol. Chem. **267,** 5882–5888.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5,** 3610–3616.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Höfte, H. (1997). Cellular basis of hypocotyl growth in *Arabidopsis thaliana*. Plant Physiol. **114,** 295–305.

Golan, A., Tepper, M., Soudry, E., Horwitz, B.A., and Gepstein, S. (1996). Cytokinin, acting through ethylene, restores gravitropism to Arabidopsis seedlings grown under red light. Plant Physiol. **112,** 901–904.

Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., and Pastan, I. (1995). Genetic analysis of the multidrug transporter. Annu. Rev. Genet. **29,** 607–649.

Gottesman, M.M., Pastan, I., and Ambudkar, S.V. (1996). P-Glycoprotein and multidrug resistance. Curr. Opin. Gen. Dev. **6,** 610–617.

Herman, E.M., Li, X., Su, R.T., Larsen, P., Hsu, H., and Sze, H. (1994). Vacuolar-type H^+-ATPases are associated with the endoplasmatic reticulum and provacuoles of root tip cells. Plant Physiol. **106,** 1313–1324.

Higgins, C.F. (1992). ABC transporters: From microorganisms to man. Annu. Rev. Cell Biol. **8,** 67–113.

Jackson, D. (1991). In situ hybridization in plants. In Molecular Plant Pathology: A Practical Approach, D.J. Bowles, S.J. Gurr, and M. McPherson, eds (Oxford, UK: Oxford University Press), pp. 163–174.

John, M., Schmidt, J., Walden, R., Czaja, I., Dulz, M., Schell, J., and Rohrig, H. (1997). Lipochitooligosaccharide-induced tobacco cells release a peptide as mediator of the glycolipid signal. Proc. Natl. Acad. Sci. USA **94,** 10178–10182.

Kammerloher, W., Fischer, U., Piechottka, G.P., and Schäffner, A.R. (1994). Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J. **6,** 187–199.

Kendrick, R.E., and Kronenberg, G.M.H. (1994). Photomorphogenesis in Plants. (Dordrecht, The Netherlands: Kluwer Academic Publishers).